Rhythms of Life
An introduction using selected topics and examples

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In memory of my teachers Erwin Bünning, Colin S. Pittendrigh und Jürgen Aschoff

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Revised Version

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Alle Schöpfung schwingt im Reigen, Freude heißt ihr hohes Lied.
Nur der Mensch will sich nicht beugen, jagt nach fremdem Glück sich müd.
Freunde, sucht den Sinn der Dinge, daß auch Freude euch durchdringe.

(v.Goethe)

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For the Reader

You shall not finish your work,
but you can not refrain from it[1]

This manuscript is based on a series of lectures I gave at the University of Tübingen. Aim of the lectures was to give examples of these rhythms, to show how interesting it is to study these phenomena, how widespread they are throughout the living world, to point out the variety of their occurrence, and how little we still know about the mechanisms underlying these rhythms.

Since this field has been and still is growing rapidly, I can offer only a limited view. I had to select out of the many observations, studies and experimental work. This selection is of course quite subjective. I tried to arrange the different chapters in such a way that the variety of topics is reflected, and yet a 'red thread' (as we say in German) connects them.

For one person it is quite difficult to put together all the many mosaic stones to one picture in which the forest can still be seen in spite of the many trees. On the other hand it is the trees which make the wood. If they are painted diffusely, the wood will be blurred. Therefore certain milestone trees are selected and described in more detail. They serve also as points for orientation and as view points.

I do realize that I have not (or not sufficiently) mentioned many interesting areas and objects. I might ask therefore the experts among the readers to join me and make her/his knowledge public. Please send illustrative material and text and point to titles of books, review articles and addresses in the Internet which I can refer to (your home-page, literature and so on). Let me know about errors, criticize and propose changes.

This book was published in English (or rather broken English – the international language of scientists), to make it available to the non-German readers. The idea is, that this publication should grow in the Internet until it has reached a certain size and quality. At that time a compact disk (or perhaps some other medium) could be produced with text, illustrations and short movie parts. It can be edited anew after some time. A CD might be a good way to give publicity to this area on rhythms in organisms among young people as a starting point for studying them more intensively. Other groups (for instance Gerda and Günther Fleissner at the department of Zoology of the University in Frankfurt, Germany[2]) are also working on such information material. My EMail address is Engelmann@uni-tuebingen.de.

For observations, studies and experiments in the area of chronobiology a book is available (http://w210.ub.uni-tuebingen.de/volltexte/2009/3790/).
It and several further books on this topic can be obtained from the Internet: http://w210.ub.uni-tuebingen.de/volltexte/2009/3790/;

[1] *Mischna, proverbs of the patrons II,16*

[2] fleissner@zoology.uni-frankfurt.de

http://w210.ub.uni-tuebingen.de/volltexte/2009/3768/;
http://w210.ub.uni-tuebingen.de/volltexte/2009/3795/;
http://w210.ub.uni-tuebingen.de/volltexte/2009/3793/;
http://w210.ub.uni-tuebingen.de/volltexte/2009/3766/;
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http://w210.ub.uni-tuebingen.de/volltexte/2009/3800/;
http://w210.ub.uni-tuebingen.de/volltexte/2009/3768/;
Topics which are treated there extensively will be referred to in this book.

Introduction to the topic

Rhythms[3] are a property of all complex systems. Engineers use much of their time to avoid that for instance motors induce resonant oscillations in the buildings or in a car. Bridges can be damaged by oscillations induced by wind or by the traffic. Therefore it was forbidden that soldiers marched abreast over a bridge. It might collapse by resonance. Rhythms are widespread in the economy and are subject to governmental and private speculations. E. R. Dewey became a millionaire by observing these rhythms and buying and selling stock shares at favorable times ([318], upper part of figure 0.1).

The way to do it is quite simple and is illustrated with the pig cycle: Selling pigs makes good money. Many farmers start to raise pigs anew or at a larger scale. For some time this works well, until the market is saturated. Now the prizes fall, until it is not worth anymore to keep pigs. As a consequence, the offers at the market go down. Prizes rise and the pig cycle starts anew. Clever farmers behave in an anticyclic way.

Similar interactions are found in a predator-prey-population. If in an area many prey animals exist, the predators are well off and can reproduce at a higher rate. The increasing predator population however reduces the prey. Finally there is not enough food left, the number of predators increases less strongly and the prey

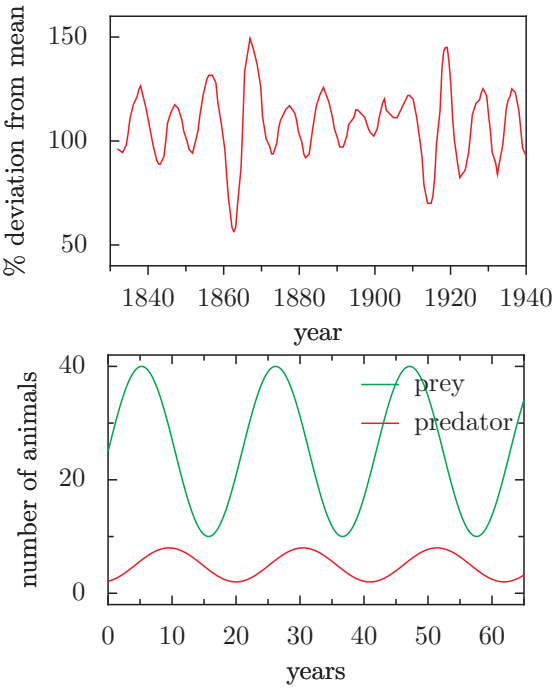

Figure 0.1: *Examples for oscillations: Top: Nine-year-rhythm of wholesale prizes, percent deviations from the moving three year mean from 1830 to 1940 (after [318]). Below: Oscillations in populations of predator and prey, model simulation, number of animals in the predator and prey population as a function of time (years). After [1536]*

[3] Rhythms are events which repeat themselves periodically. Physicists use the expression oscillation.

population can recover. For a predator-prey-model see http://w210.ub.uni-tuebingen.de/volltexte/2009/3790/.
There are programs which graphically display the interplay between the sizes of the populations of predator and prey.

Numerous rhythms are also found in organisms. This is nothing special, since they -being complicated systems- tend to oscillate. We find oscillations in the metabolism, in physiological processes, in reproduction and in behavior. Some of these oscillations are adverse and the organisms try to avoid them. However, in many cases organisms make use of them. Chemical reactions or physiological processes are often more favorable for the organisms if they occur in a rhythmic way. This is true especially for those events which depend on rhythmic environmental conditions: Photosynthesis can occur only during the light. Nitrogen fixation of some *Cyanobacteria*, on the other hand, occurs in the dark, because an enzyme of this reaction is inhibited by oxygen. During photosynthesis oxygen is produced. Therefore a daily rhythm takes care, that the two incompatible processes of photosynthesis and nitrogen-fixation are separated in time (see page 127).[4]

The properties of rhythms will be demonstrated in examples and it will be shown how to describe and record rhythms. In figure 0.2 the most important terms such as period length τ, phase Φ, amplitude A and phase relationship to the light-dark-cycle Ψ are described. The spectrum of rhythms in organisms ranges from *ultradian rhythms* in the high frequency part (milliseconds to hours) via *daily rhythms* (so called circadian rhythms), *tidal rhythms*, *monthly-* and *fortnight rhythms*, *annual rhythms*, up to even longer rhythms with periods of several years. To these last mentioned belong the rhythms between populations of predators and prey, but also developmental cycles of organisms ('He is so old that he has seen the bamboo flowering twice', Asian proverb; [976]; figure 0.3 and 0.4).

These rhythmic events in organisms, their causes and significance is studied by *chronobiologists*. Chronobiology deals with the (rhythmic) time structure of organisms, populations and ecosystems.

[4] In certain *Cyanobacteria* these mutually incompatible processes are separated from each other in space: Nitrogen fixation occurs in 'heterocysts´

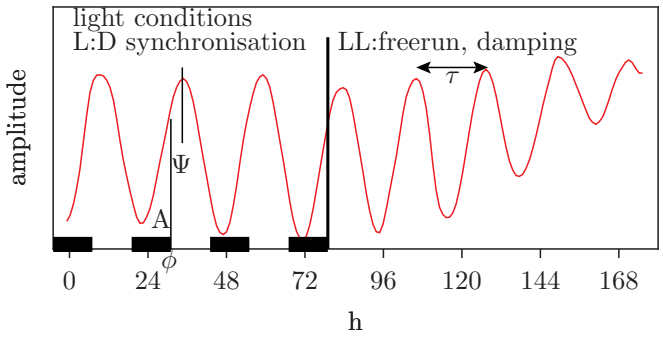

Figure 0.2: *Description of oscillations: An organism containing an endogenous (internal) oscillator is synchronized by the light-dark-changes (LD) of the environment (in the example given by 12 hours light and 12 hours darkness). Its period length is 24 hours. Afterward continuous light (LL) is applied. Now the organism shows 'free run´ with a period length which is shorter than 24 hours. Furthermore in this particular case the rhythm damps under continuous light. Phase ϕ is a time point on the curve, period length τ is the time span between corresponding phases such as two successive maxima of the oscillation, amplitude A is generally used to denote the y-axis of the different phases ϕ of the oscillation, but also for characterizing the maximal y-value (to be exact, this should be called 'maximal amplitude´), and phase relationship ψ is the time span between the maximal phase and some external event such as the end of darkness and begin of the light period)*

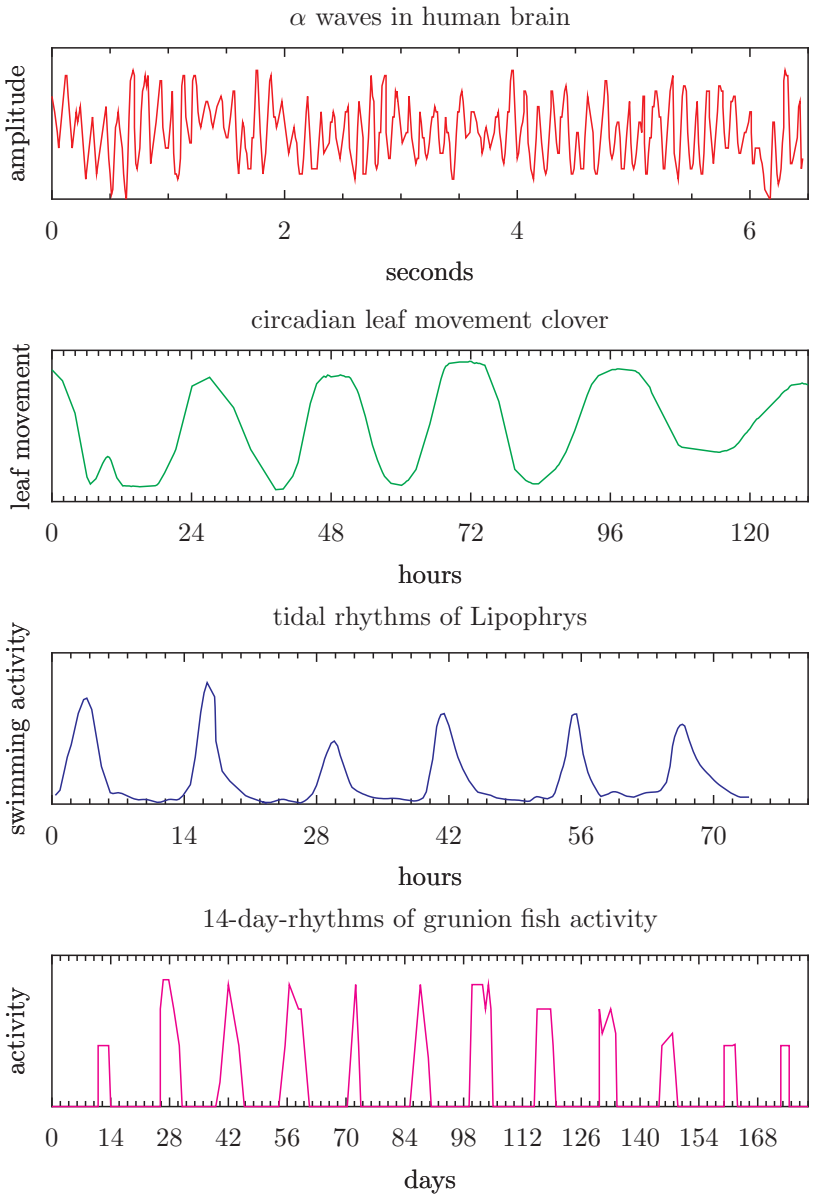

Figure 0.3: *Spectrum of rhythms in organisms (note the different time axes): a: As an example for ultradian rhythms the firing of a nerve cell, b: As an example for daily rhythms the day- and night position of clover leaves, c: As an example for tidal rhythms the swimming activity of a coastal fish* Lipophrys pholis *(after [495]), d: As an example for a fortnight rhythm the activity of grunion fish* Leuresthes tenuis *(after [1525])*

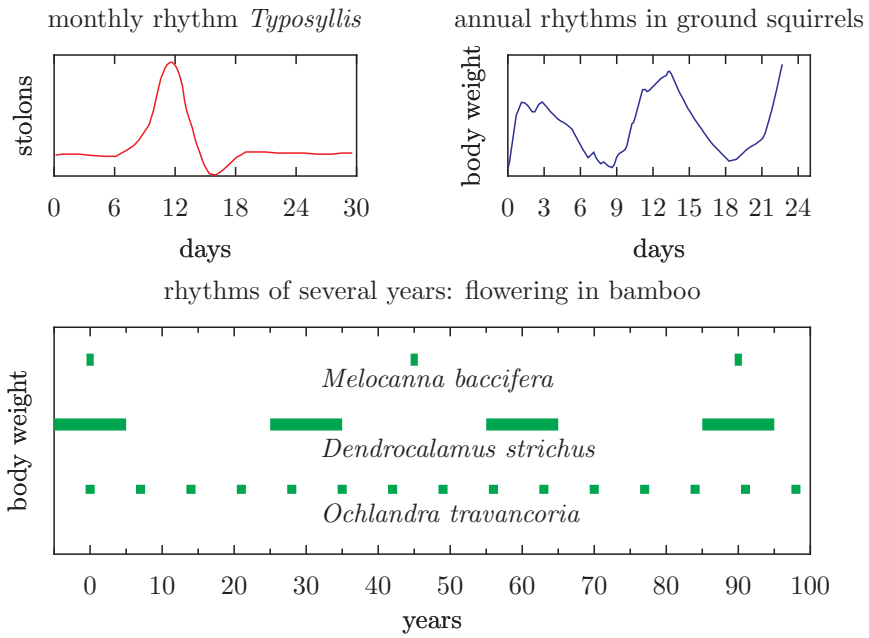

Figure 0.4: *Top left: As an example for monthly rhythms the separation of stolons of the polycheat* Typosyllis prolifera *(after [454]), top right: as an example for annual rhythms the change of body weight in the ground squirrel* Spermophilus lateralis *(after [542]), bottom: as an example for even longer rhythms with periods of several years the flowering of bamboo (data from [976])*

Overview of this book

First we will get to know examples of rhythms. Among the ultradian rhythms a chemical oscillator, the glycolysis-oscillator of the yeast, the gravitropic pendulum, transpiration rhythms in oat, the lateral leaflet movement of the telegraph plant, the circumnutation in plants and the REM-sleep of mammals are described.

The circadian clock of man controls sleep and wakefulness, activity and body temperature and numerous further events in the body. The circadian system can be disturbed by shift work and jet-lag. In extreme cases this can lead to diseases, or diseases can influence the circadian system. *Chronomedicine*, *chronohygiene* and *chronopharmacology* are concerned with this.

How these rhythms function and how they are influenced can be better studied in animals as compared to man. Among mammals, hamster and mice are especially suited for those studies. The locomotor activity and body temperature can be recorded continuously. Since both processes are controlled in a circadian way, they are useful as 'hands' of the circadian mechanism, like the hands of a clock that tell us there is a clockwork behind. How does this circadian system function? Does it consist of a main oscillator, which governs all the 'hands' in the body? Or are several, or even many oscillators involved, which are controlled by a main oscillator? How do they interact with each other if there are more than one? Where are these oscillators localized?

In mammals it was found that such a center is located in the suprachiasmatic nucleus (*SCN*) of the hypothalamus in the brain. Furthermore the *pineal* organ and a hormone of this gland, *melatonin*, play an important role. Due to the pineal and melatonin the information 'darkness' is transmitted. This information controls *photoperiodic reactions*, which are used by many mammals of the temperate and higher latitudes to adapt to the seasonal changes. The light-dark-cycle synchronizes the circadian system. The light is received by the retina of the eyes. In other vertebrates further *photoreceptors* are involved.

Circadian rhythms are not only found in man and other mammals, but throughout the whole living world.

Unicellulars possess circadian clocks, such as the marine diatom *Lingulodinium*. This unicellular alga shows a dim glow, which can often be observed during nights on the surface of the oceans. How this bioluminescence is brought about and controlled rhythmically is well studied. *Lingulodinium* displays furthermore a rhythm in cell division and aggregation. Further circadian rhythms were found in the unicellular alga *Acetabularia.* Even prokaryotes possess a circadian rhythm. It controls among others the photosynthesis and nitrogen fixation in *Cyanobacteria*.

In higher plants especially the circadian rhythm of *photosynthesis* and *transpiration*, the rhythm of *leaf movements*, *division* and *growth* have been studied. Plants of arid areas use daily rhythms to survive. They

are able to fix carbon dioxide with the help of the *CAM-metabolism* during the night and are therefore able to keep their stomata closed during the day, thus saving water. The activity of a key enzyme of the CAM-metabolism, namely the PEP-carboxylase, is modulated in a daily fashion.

One of these CAM-plants is *Kalanchoe blossfeldiana*. It has flowers which open and close in a daily fashion. The mechanism is simpler than the one which controls quite a number of leaf movements where special joints are used. The clock which controls the petal movements of *Kalanchoe*-flowers has been studied intensively and some of the results are presented.

Many other plants open and close their flowers in a daily rhythm and/or produce *fragrance* at certain times of the day which attracts insects, birds and bats. This insures pollination and prevents self pollination of the plants. The plants as well as the pollinators use adaptations in respect to the time structure. Insects which pollinate these plants are themselves equipped with circadian clocks. They help them in orienting in time and space. Time sense of bees and *sun compass orientation* belong to this category.

Examples for *tidal rhythms* are mainly found in the coastal areas of the sea. Organisms in this biotope possess also clocks which run according to the moon. There are *monthly rhythms* (period length of 28 days) and *fortnight rhythms* (period length of 14 days).

Annual rhythms are wide-spread among organisms. They occur also if organisms are kept under conditions shielded from rhythmic changes in the environment. They are found in seeds of plants, in insects, snails. Such rhythms have been observed even in a unicellular alga. In birds annual rhythms are especially well studied and they are closely connected to migration. In mammals reproduction and hibernation are controlled by an annual rhythm. Even in man there seem to be events which are influenced by an annual clock. The significance of annual rhythms will be highlighted.

This annual clock is often timed to the season photoperiodically. It has to be synchronized with the external world, if this clock runs endogenously (based on an internal oscillator). For this purpose the length of the day is measured, which changes during the course of the year in a predictable way. Day-length is, especially in areas further away from the equator, the most reliable time cue for the season of the year. The *photoperiod* determines, whether tubers, bulbs or onions are formed, whether certain plants become succulent, whether seeds stay in a dormant stage or begin to germinate, whether plants are induced to flower, whether insects go into a resting stage, whether birds and mammals will reproduce.

In a further chapter we will study the clocks of *Drosophila*: Its hands, its localization, and its mechanism of control. Here the mechanism of the circadian system has been investigated intensively in the last years using also molecular biological methods.

From the nerves of eyes of certain marine snails circadian action potentials can be recorded even if the eyes with their nerves are isolated from the animal. The oscillators responsible for the rhythm are situated in the basal cells of the eyes.

Fungal rhythms and *coral clocks* are reported on in the chapters to follow. The bread mold forms spores only at certain times of the day. It is based on a circadian

rhythm and has been studied using molecular biological techniques also.

The foot of a coral ('epithok') is formed by layers deposited in a daily and an annual rhythm. By counting them and relating them to each other it has been show that the days in former epochs of the earth were shorter and that therefore a year had more than 365 days.

The *significance* and *selective advantage* of all these rhythms for organisms and their evolution will be treated in a further chapter. It will also be a matter of interest how this area will develop in the future and what might be the focus of future research.

Finally there is a collection of *special topics* in section 20, where I have tried to consolidate or illustrate certain parts (incomplete or planned only: Here your contribution is especially welcomed).

In different parts of this book there are also links to experiments which can be performed in courses and which have been described in a course book (http://w210.ub.uni-tuebingen.de/volltexte/2009/3790/).

1 Ultradian rhythms

Ultradian rhythms are periodically occurring events in the range of minutes to hours and widely distributed among organisms. But even chemical reactions might oscillate as can be demonstrated with the Belousov-Zhabotinskii-oscillator. This reaction shows oscillations in time, but also wave patterns of chemical activities.
In yeast an ultradian oscillation of NADH-fluorescence can be demonstrated. It is based on oscillating events during glycolysis consisting of feedback mechanisms between the individual reaction steps.
Sun flower seedlings and other plants exhibit a so called gravitropic pendulum movement after stimulation by gravity. If a pot with such a seedling is turned sideways for some time and put back in its original position again the hypocotyl oscillates under suitable conditions in the plane, in which it was stimulated by gravity.
Circumnutations are often observed in growing plants: The tips of the seedlings do not simply grow upward, but move during elongation in a circular or elliptic way. We will look more closely at these movements in the hypocotyl of Arabidopsis thaliana.
Transpiration of grasses via the stomata can also occur rhythmically. Water loss of the leaves as well as water uptake by the roots is rhythmic.
In the telegraph plant Desmodium gyrans *the lateral leaflets show fast up- and down- or turning movements which are brought about by turgor changes in special joints.*
Finally the REM-sleep of mammals will be presented as a further example of ultradian rhythms.

1.1 Chemical Oscillators

Chemical oscillators are, in a strict sense, not a topic of this book ('Rhythms of life'). But they demonstrate in an impressive way how even relatively simple chemical systems can oscillate. Therefore we will briefly describe one of these chemical oscillations, the Belousov-Zhabotinskii-reaction. This reaction was discovered by [81] and studied in detail by [1607]. According to the laws of thermodynamics, in all spontaneously occurring chemical reactions in a homogeneous, closed system the free enthalpy[1] decreases. For this reason oscillations should not be expected to occur. However, at certain conditions the concentration of intermediates can oscillate around the expected values in a stationary state (figure 1.1). For this to happen the reaction must not be in equilibrium.

The reaction consists of two main reactions A and B, which influence each other only slightly, since A consists of reactions between ions and molecules with paired electron spins (singlets), B consists of reactions between radicals. Whether A or B dominates depends on the concentration of Br^-. At high concentrations A dominates, at low concentrations B. A uses Br^-;

[1] a thermodynamic property of a system defined as H=U+PV (H enthalpy, U internal energy of system, P pressure exerted on the system by its environment, V volume of the system)

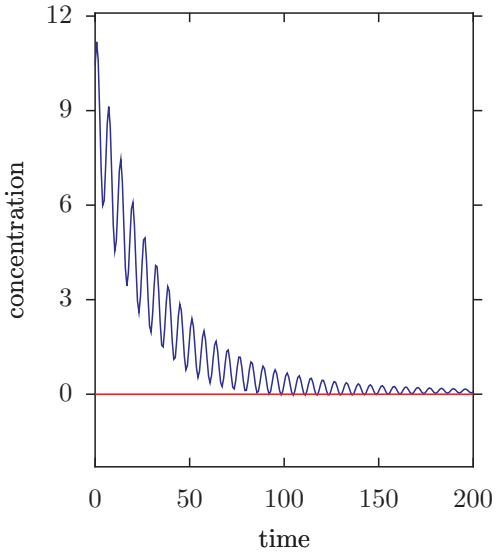

Figure 1.1: *Course of a chemical oscillation in a closed system, before the equilibrium (horizontal line) is reached. On the y-axis the concentration (arbitrary unit) of an intermediate is plotted (after [303])*

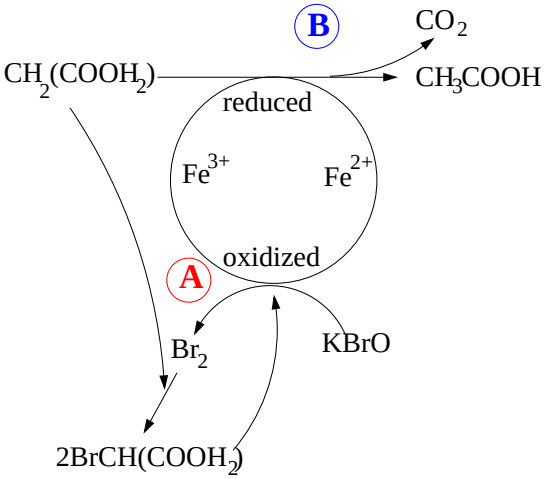

Figure 1.2: *Reaction scheme of the Belousov-Zhabotinsky-reaction with the two main reactions A and B. A: Bromide and bromate form, together with malonic acid, bromine malonic acid. The ferroin is first blue, due to the trivalent iron (bromate oxidizes Fe^{2+} to Fe^{3+}). If bromate disappears, Fe^{2+} is formed. The ferroin turns red. B: Bromine malonate is concentrated enough to reduce Fe^{3+} to Fe^{2+}. Acetic acid is formed, CO_2 (gas bubbles!) and bromide, which inhibits reaction A: The formation of bromine malonate is stopped, reaction B comes to a halt. After [1572]*

in this way the reaction B is induced. B produces Br^-; in this way A is induced (figure 1.2). For more details see [427] and [1572]. The same chemical reactions are responsible for a wave pattern. It is found after pouring the solutions in a flat dish and keeping it undisturbed. In this case the diffusion of bromine ions plays a role, because the solution is not stirred (figure 1.3, movie [641]).

As in most of the ultradian rhythms the period length of this chemical oscillator depends strongly on the temperature of the solution. At a 10^0C higher temperature the period is half as long.

For experiments with chemical oscillators see http://w210.ub.uni-tuebingen.de/volltexte/2009/3790/.

1.2 Glycolysis-oscillator

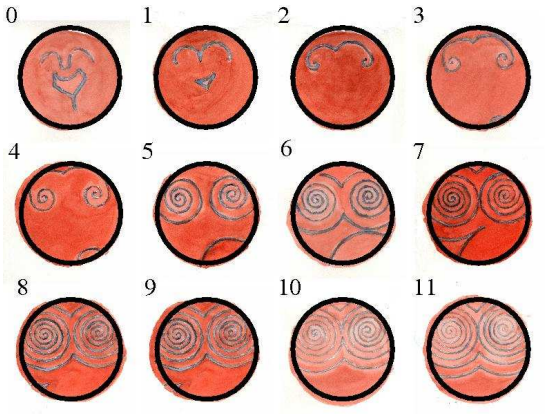

Figure 1.3: *Belousov-Zhabotinsky-reaction as a wave pattern in a Petri dish. First the solution is red. By a disturbance at one place bromine ions are used up, bromate oxidizes Fe^{2+} to Fe^{3+} (blue). Bromine ions diffuse into the surrounding and in this way the red area turns into a blue ring. If the bromine malonate has reduced Fe^{3+} (bromine ions are formed), the area turns red again. The pictures 0 to 11 show the changes in 60 second intervals. After [1572]*

Cells can produce energy in three ways: By photosynthesis, by respiration, and by glycolysis. Glycolysis is used by organisms which live without oxygen such as yogurt-fungi, bacteria in pickled cabbage, parasitic worms, red blood cells, diving vertebrates.

During glycolysis glucose is converted to pyruvate and ATP is produced as a carrier of energy. Nine different enzymes participate in this reaction chain (figure 1.4). [347] found that glycolysis in yeast does not always proceed uniformly, but is rhythmic under certain conditions. During the fermentation of glucose by yeast (that is, without oxygen) alcohol is formed. The biochemical reactions involved in glycolysis-oscillations of the yeast *Saccharomyces* are well known. If the different enzymatic reactions are connected with each other as equations, oscillations will be found in certain reaction steps. Oscillations are indeed found in the experiment, if glucose as a substrate is added to a suspension of yeast cells ([102]). The easiest way of recording them is by measuring the fluorescence of NADH (figure 1.5). Depending on the conditions the period length varies between 2 and 70 minutes and is strongly dependent on temperature.

To observe this phenomenon the yeast suspension is nutrient deprived (no sugar offered). After NADH fluorescence has dropped to low values, glucose is added, until the concentration is 100 mM. Afterward KCN is added which prevents the cells from having oxygen at their disposal. Glycolysis, which is now starting, proceeds in a cyclic way with a period length of about one minute at 20^0C.

The oscillations are found in a criti-

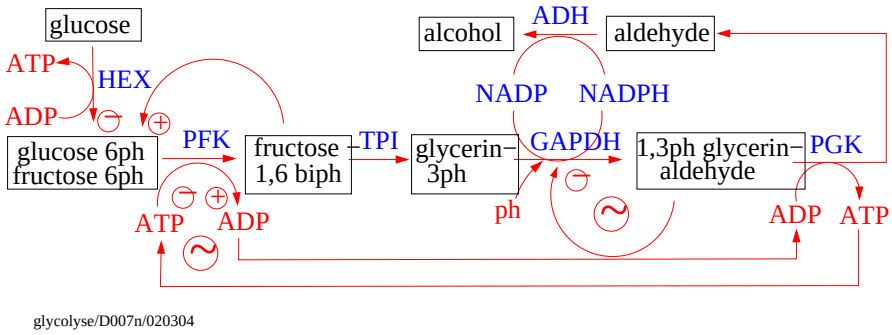

Figure 1.4: *Course of glycolysis in yeast and feedback of substrate on enzymes. With ~ the positions are marked at which oscillations occur. →: flow. Broken arrow: Feedback path. +: Activation, -: Inhibition. HEX: Hexokinase, ATP: Adenosin triphosphate, ADP: Adenosin diphosphate, PFK: Phosphofructokinase, TPI: Triose phosphate-isomerase, GAPDH: Glycerine aldehyde/-phosphate-dehydrogenase, ph: Phosphate, PGK: phosphoglycerokinase, ADH: Aldehyde-dehydro/-genase, NADP: Nicotinamide-dinucleotide-phosphate. After [227], [362]*

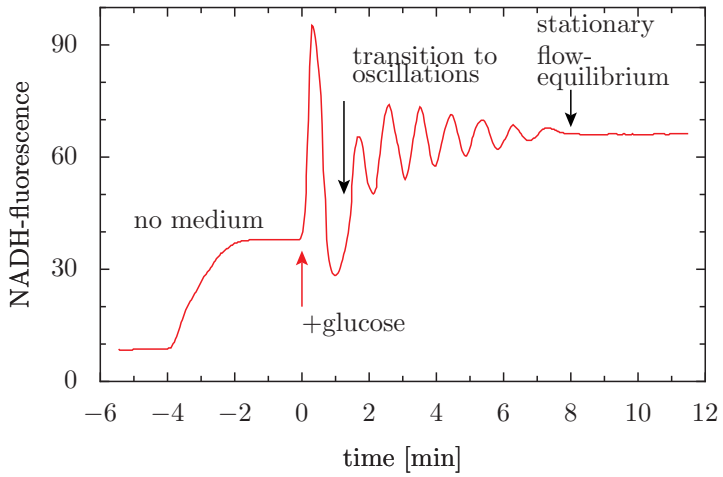

Figure 1.5: *Induction of glycolysis-oscillation in a yeast suspension. At time 0 (red arrow) glucose was added to the nutrient deprived suspension. The stationary flow equilibrium is reached after an oscillatory transient. The fluorescence of NADH was measured and used as an indicator of glycolysis. After [18]*

cal range of the flow rate, where feedback occurs during the course of the reactions (figure 1.4). The aim of the glycolysis is the production of ATP. ADP controls the activity of the phosphofructokinase (PFK) by binding to a specific receptor of the enzyme. By this the form of the enzyme is changed and it works hundred times faster. At lower ADP-concentrations (i.e. a higher ATP-content) glycolysis is inhibited. In this way the oscillations are brought about.

With oxygen-pulses the phase of the glycolysis-rhythm can be shifted. If a defined pulse is given in a critical phase, the rhythm disappears. This indicates that the limit cycle describing the oscillating system is based on two state variables only ([1570]).

The glycolysis-oscillations can be observed also in cell free extracts ([629]).

For the timing and coordination of metabolic events some organisms use also ultradian clocks which - like circadian clocks - are *temperature-compensated*. Their frequency is, however, higher and they can not be synchronized by external time cues in a 24 hour measure. If one watches the movement of individual *Paramecium* under the microscope, periods are found during which these unicellulars swim more or less straight with rare deviations in other directions. After a certain time the swimming pattern changes. Now only short distances are covered by straight swimming and the direction is changed frequently. This change in behavior shows a period of 45 minutes. The same periods are found at higher and lower temperatures ([911]). This particular ultradian rhythm is thus, like circadian rhythms, temperature compensated. It points to a function as a clock. In the meantime further examples for temperature compensated ultradian rhythms have been reported (overview [912]).

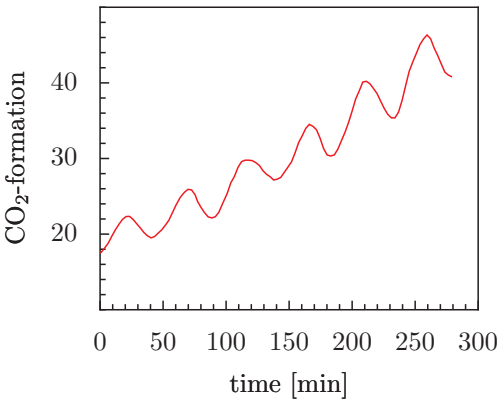

Figure 1.7: CO_2 *formation in a fermenting* Schizosaccharomyces pombe *culture at* 30^0C *after 4 temperature-cycles of 30 minutes* 30^0 *and 15 minutes* 26^0C *(not shown). Measurements every 5 minutes in three different and independent experiments. The data were averaged and smoothed. After [778]*

For instance, in fast growing cells of *Schizosaccharomyces pombe* an ultradian clock controls cell division. A rhythm with a period length of 40 to 44 minutes is found (figure 1.6). This rhythm is temperature-compensated and independent of growth rate ([777]). It continues for at least 18 hours without damping. This is an indication for inter-cellular communication. With the same period the CO_2 formation fluctuates in fermenting (figure 1.7) and also in respiring cultures, as does the O_2 uptake and the acidification of the culture medium. If cell division is blocked, the other three rhythms continue. They are therefore not a direct consequence of the cell division rhythm. Here too an ultradian clock seems to exert a general control of metabolic events ([778]).

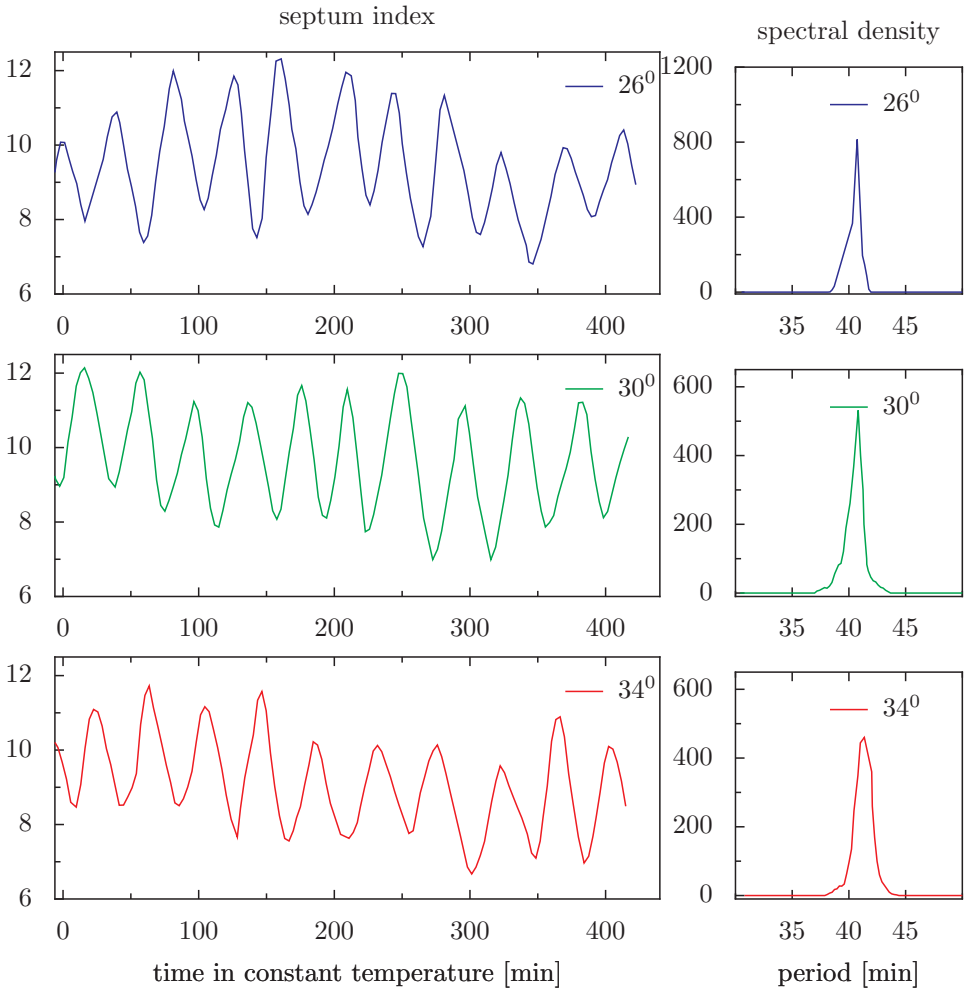

Figure 1.6: *Cell division in* Schizosaccharomyces pombe *cultures. Cells were first synchronized by temperature cycles of 30 minutes with* 33^0C *and 15 minutes* 27^0*. Afterward the cultures were transferred into constant temperature conditions of* 26^0*,* 30^0 *or* 34^0C*. The septum-index was determined as the percentage of cells which have formed a septum. The time series analyses using maximum-entropy-spectral-analysis procedures (MESA) are shown at the right of the curves (spectral density against period length). They show at all three temperatures the same period of about 40 minutes: Period length thus does not depend on the temperature of the medium. After [777]*

1.3 Circumnutation in plants

Plants react very accurately to gravitational forces in order to grow adequately and to orient themselves in space ([521], [710]). However, in growing upward, elongation does not occur normally as a uniform stretching. Instead the acute growth zone migrates around the periphery. This leads, seen from above, to circular, elliptic or pendulum like movements of the corresponding tip of the organ. Seen from the side the tip of the organ describes a helix. In tendrils of climbers these circumnutations are especially pronounced. The plants try in this way to find a hold. Circumnutations are wide spread among climbing and non-climbing dicots, monocots, gymnosperms and even among fungi and bacteria. Roots can show these nutational movements too ('root waving', [1304]). Overviews are given by [41], [712], [168]. Newer results in [1108], [1472], [1357].

Circumnutations are always correlated with growth. Beyond a growth rate of 0.5 mm no circumnutation is found in *Periploca graeca* ([993]). The period length depends on temperature and on the object. It is normally between 15 minutes and 5 hours. Some species possess circumnutations with different frequencies (*Sicyos*, *Passiflora:* [520], *Phaseolus:* [590], *Arabidopsis:* [1342]). The excursions are just fractions of millimeters in *Arabidopsis*, but up to 1.5 m in *Hoya carnosa*. Depending on species and oscillation there are preferred directions to the right or left ('chirality'). Oscillations with different periods can overlap each other.

The hypocotyl of *Arabidopsis thaliana* shows a whole spectrum of circumnutations with 'short period nutations' and 'long period nutations'. Their underlying mechanisms will be dealt with more closely in chapter 20, page 411.

The growth of the hypocotyl of *Arabidopsis thaliana* is furthermore under circadian control, as can be seen under constant conditions. There are periods with low growth rates and those with high growth rates. Since ultradian circumnutations occur only during growth, they must be modulated by the circadian rhythm. In *Arabidopsis* there are other events which are controlled in a circadian way ([1010]).

Growth and circumnutations of *Arabidopsis thaliana* (and other plants) can be recorded and analyzed by using imaging procedures.

Many other plants show circumnutations. Well studied are, for instance, the pendulum movements of sunflower seedlings grown in the dark.

1.4 Gravitropic pendulum

The ability of plants to grow upward again after having been thrown down by heavy rain or storm as well as the climbing and twining is known since a long time ([1020]). [290] have pointed out how frequently circumnutations are found even in non-twining plants. Linné has introduced the term circumnutation. Today the whole spectrum of movements brought about by unequal growth, i.e. circular, elliptic and pendulum like movements, are called circumnutation ([680]).

If sunflower seeds germinate in the dark or under red light (physiological darkness) and the hypocotyl has grown to about 5 to 6 cm, turning the seedling by 90^0 for e.g. 30 minutes induces a gravitropic pendulum movement (experiments described in http://w210.ub.uni-tuebingen.de/volltexte/2009/3790/).

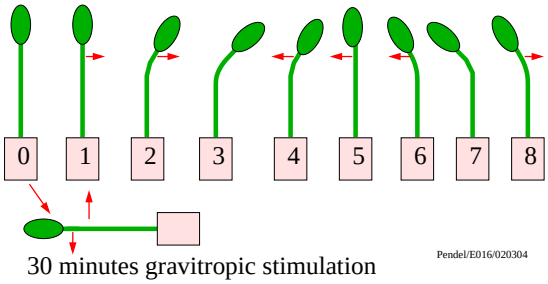

Figure 1.8: *A sunflower seedling is put horizontally for about 30 minutes (0) and afterward back to vertical (1). The gravity stimulus (red down arrow) leads after a certain time to a reaction: The tip of the seedling bends (2) and bends further (3). In the state of maximal bending, however, the difference of the hormone concentrations at the two sides is gone. A new gravitropic stimulus stimulates the hypocotyl anew. It bends now to the other side (4), overshoots (5) and bends to the other side (6), until a new gravitropic stimulation occurs (7) with a counter-reaction (8). The red horizontal arrows indicate the direction of bending. According to [385]*

Putting the plants back in a vertical position, the tip bends away from the plumb line. After maximal bending a counter-movement in the other direction begins. This pendulum movement continues for some time (figure 1.8). It is even amplified if the hypocotyl elongates further during physiological darkness. Finally the strongly etiolated stem might even collapse.

1.4.1 Physiology of gravitropism

For more recent reviews of the physiology of gravitropism see [1303], [1451], [557]. Gravitropism might occur as ortho- (in the direction of the gravity), dia- (perpendicular to the gravity axis), plagio- (diagonal downward) and agravitropism (against the gravity). Roots grow in the direction of gravity or diagonal to it. The root cap perceives the gravitropic stimulus. Stems grow negative gravitropic.

Plant organs can react very sensitively toward gravity. A stimulus duration of 0.7 seconds is already sufficient ([1379]). There are different hypotheses how gravity is transformed into a physiological reaction ([1303], [1451]). According to the statolith theory specific cells, the statocytes, are responsible for perceiving the gravitropic stimulus. They possess amyloplasts with starch grains or vesicles containing $BaSO_4$ (*Chara*) (‘statoliths´). A number of arguments are in favor of this theory ([1378]). Without starch grains there should be no gravitropism. However, even starch free mutants of *Arabidopsis* do still react ([210]). Amyloplasts are, however, effective even without starch grains ([780]). Decisive is the density. It is for starch 1.3, as compared to 1.0 of the cytosol and the nucleus. Size is also important. Too small and too light particles show Brownian movements and are not suited to serve as gravity receptors.

An alternative to the statolith hypothesis was proposed by [1154]. In this ‘plasmalemma central control model´ stretch activated calcium channels are clustered around attachment centers which connect the cytoskeleton and the cell wall. The channels open in response to tensions at the membrane by the protoplast, the cytoskeleton or the cell wall. The tension is induced by gravity.

After perception the gravitropic stimulus must be transduced in order to lead to a differential growth reaction. Lateral redistribution of auxin caused by gravistimulation ([235], [1552]) or changes in auxin sensitivity ([1302], [412]) were assumed to lead to this reaction. However, gravitropic

experiments in coleoptiles by [354] favor a new model: Retention of a loosening factor to infiltrate the walls inhibits growth on the upper side of a coleoptile put horizontally, whereas the lower side is infiltrated by the loosening factor. As a consequence, the coleoptile bends upward.

Depending on whether it is a positive (for instance in roots) or negative (for instance in shoots) gravitropic reaction the upper or the lower side of the organ grows more strongly.

1.4.2 Recording of the movement and analysis

The pendulum movement can be recorded with an imaging system (http://w210.ub.uni-tuebingen.de/volltexte/2009/3790/). During and after recording the movement can be plotted in a time diagram (figure 1.9). With time-series analysis procedures (see section 20.20) the period lengths of this oscillation can be determined.

1.4.3 Exogenous or endogenous?

The movement can be described with a feedback model ([711] and figure 1.10).

To decide whether we are dealing with an exogenous or an endogenous oscillation, an experiment was performed as shown in figure 1.11 (Johnsson in [387]). The result of this experiment suggests, that the pendulum movements of sunflower seedlings are apparently of an exogenous nature. A deviation from the plumb line acts as a gravitropic stimulation, which leads to a lateral growth of the hypocotyl. On the counter-side a new gravitropic stimulation occurs, which after some time bends the hypocotyl in the other direction. In this way the pendulum movement is brought about.

However, experiments in a space shuttle have shown that under microgravity conditions bending reactions are still observable ([168]). This points to an endogenous oscillator, which leads to an unequal, but coordinated growth. Such movements are long known as circumnutations and we have gotten to know them already in the preceding section. A re-analysis of the data from the space experiment has shown, that these movements are composed of oscillations with different periods ([52]).

1.5 Transpiration rhythms in oat

Water is extremely important for all organisms. Cells and tissues have a high water content and all biochemical reactions proceed in an aqueous milieu. Plants in arid environments protect themselves therefore by a water impermeable cuticle against water loss. On the other hand plants need CO_2 for the synthesis of carbohydrates. Therefore stomata were 'invented´ during the evolution of terrestrial plants. Depending on external and internal conditions the water content and the air exchange can be regulated by the stomata. Additionally stomata play a role in temperature regulation. The cooling effect of evaporation reduces high temperatures of the plant body. Finally, transpiration is needed also for acquiring minerals by water uptake from the soil, although this is not essential.

Transpiration is a process in which the stomata can be opened and closed in a complicated control mechanism (figure 1.12). The mechanics, control and physiol-

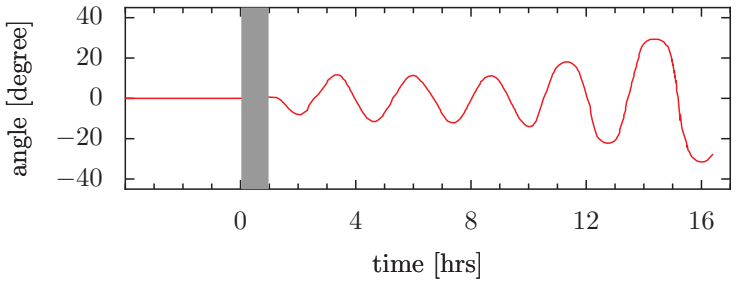

Figure 1.9: *Time course of the gravitropic pendulum of a sunflower seedling as shown in figure 1.8. At time 0 the seedling was exposed to a gravitational stimulus by turning the flower pot by 90° for one hour (black column). After returning the pot in its original position, pendulum like movements are observed*

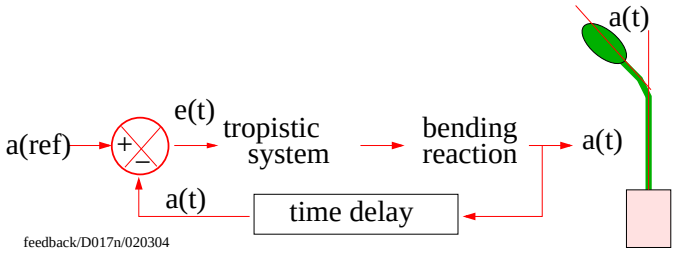

Figure 1.10: *Feedback model of the gravitropic pendulum. A reference value ('vertical growth' a_{ref}) is compared with the actual value $a(t)$. If both values differ, an error signal $e(t)$ occurs. It is amplified by a tropistic system and weighted and compared with the reference value after a time delay. At the right the current angle $a(t)$ is explained as the deviation from the plumb line. After [711]*

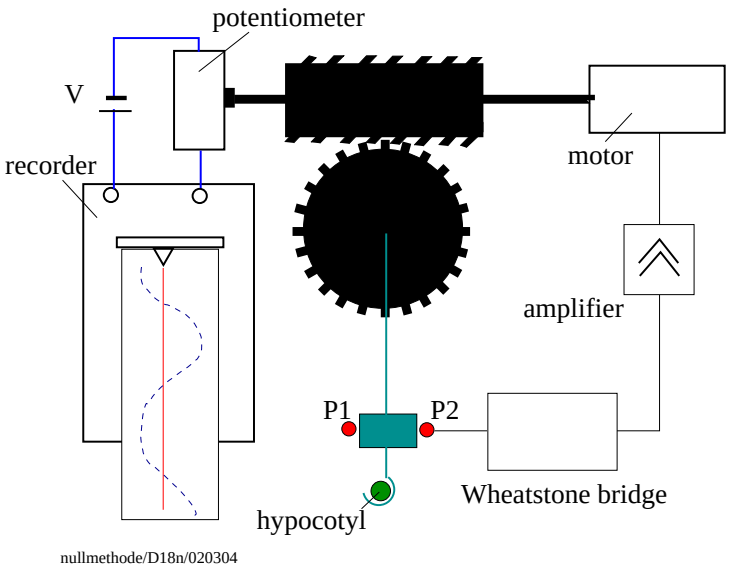

Figure 1.11: *The gravitropic pendulum movement of sunflower seedlings is recorded with a compensation method. A wire (steel blue) is fastened to a gear wheel and ends in a wire loop. Before the loop an aluminum foil (steelblue) is fixed to the wire. The loop surrounds the upper part of the hypocotyl of the seedling, which was put horizontally for a certain time before. If the tip of the seedling moves due to this stimulation, light emitting diodes (P1, P2) record the slightest deviation from the plumb line. If, for instance, the tip has moved somewhat to the left, the light emitting diode P1 is covered partly by the aluminum foil and therefore its electrical resistance changes. The signal is transferred via a comparator (Wheatstone-bridge and amplifier) to the motor. The motor turns the worm screw with the wire a bit, until the aluminum foil is not covering the light emitting diode anymore. In this way the tip of the hypocotyl is always kept straight. Furthermore a variable potentiometer passes the turns of the motor for compensating the tendency to move to a voltage recorder. The curve reflects the tendency of the hypocotyl to bend. If the pendulum movement is caused by an endogenous oscillator, the tendency of the seedling to oscillate should be measurable with this method (blue dashed curve). The recorded (red) curve shows, however, no tendency to oscillate, if the hypocotyl is kept straight. Thus the pendulum movement is exogenously caused. After Johnsson in [387]*

ogy of stomatal movements are well studied (textbooks in plant physiology such as [1303]). Stomata open because water is taken up. This is the result of K^+ accumulating in the guard cells. Their osmotic potential becomes more negative. Light induces K^+ uptake into guard cells, which in turn opens the stomata. Darkness leads to closure. The K^+ is supplied from the adjacent epidermal cells. It is not just pumped in, but exchanged against H^+. H^+ is supplied by organic acids, mainly malic acid. Starch and other carbohydrates in the guard cells are the sources of malate production. Increasing concentrations of the organic acids are also causing the osmotic potential to become more negative (see [1605]).

Abscissic acid is produced under water stress (for instance of the roots) and closes stomata by lowering cell turgor.

At least two feedback loops control the movements of guard cells (see figure 1.12). When CO_2 decreases in the intercellulars of the leaf tissue and consequently in the guard cells, K^+ enters them and the stomata open. CO_2 can now diffuse in and be used for photosynthesis. This constitutes the first loop. The second loop is shown under water stress, ABA appears and the stomata close. The plant is protected against excessive water loss. Both loops interact. The different factors affecting transpiration are shown in figure 1.12. Thus stomata have been delegated the task of providing food while preventing thirst ([1203]).

Different types of stomata exist. In figure 1.13 the *graminaean type* in oats is shown. It consists of two guard cells, which are arranged like two dumbbells and which open and close the stomata according to the degree of swelling. To view the movements of different types of stomata, movies are available (for instance [1488]).

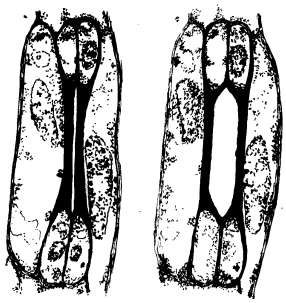

Figure 1.13: *Stoma of an oat leaf with two dumbbell-shaped guard cells surrounded by kidney shaped auxiliary cells. Stomatal pore (center) closed under conditions where the bulbous ends are shrunken and therefore the heavily thickened walls joining them touch each other (left). Swelling of the vacuoles in the bulbous ends draws the thickened walls apart, the stoma opens (right). Drawn by the author after an electron micrograph of [1127]*

Many plants exhibit rhythms in their water regulatory system. This shows up for instance in a periodic water loss of plants via the stomata (transpiration). Ultradian and circadian transpiration rhythms are known. With a suitable device one can measure transpiration rhythms for instance in the primary leaf of oats (figure 1.14). The oscillations can be described with a feedback model (figure 1.15).

To find out more about the underlying mechanisms it was tried to influence period length of these ultradian rhythms in transpiration by using different substances. Theophyllin slows the rhythm already at very low concentrations ([721]). Heavy water, Li^+, Mg^{2+}, Ca^{2+} and La^{3+} likewise lengthen period ([163], [162], [164]).

The water uptake of the roots is not

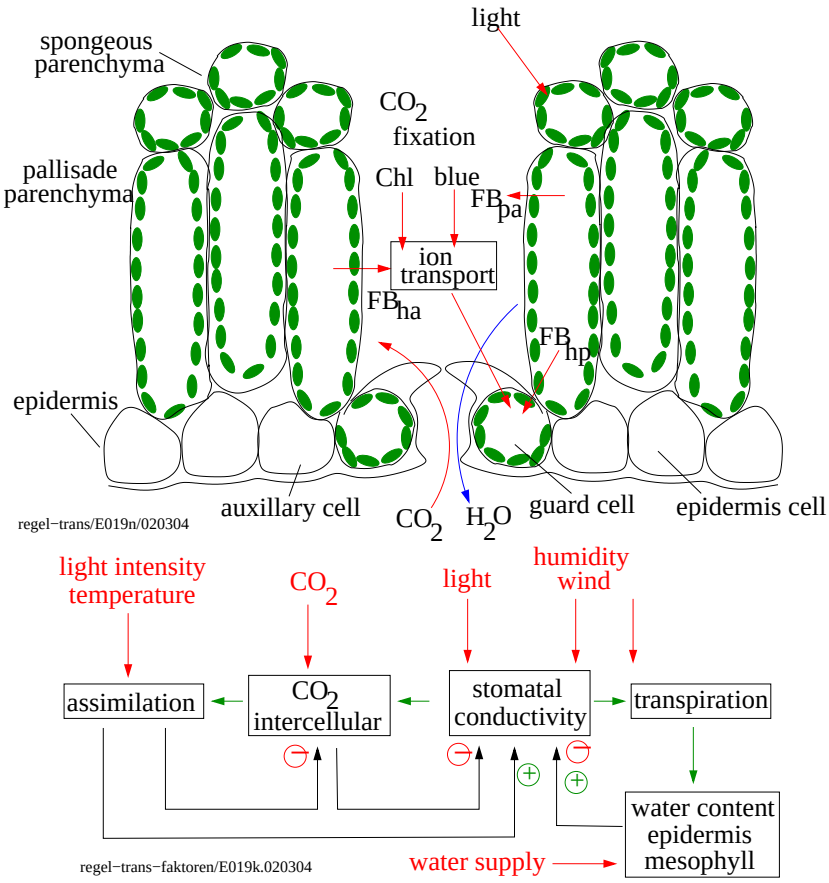

Figure 1.12: *Upper part shows schematic cross section trough the tissue of a leaf and the light effects on gas exchange. The opening width of the stomata and thus transpiration are controlled by several factors via the ion transport in the guard cells: By light directly (blue wavelength range) and via chlorophyll (Chl) of the spongy and palisade parenchyma indirectly. Different feedback loops influence the stomata: A direct hydro-passive feedback RK_{hp} of the tissue water. Furthermore a hydro-active feedback RK_{ha} from the tissue water via a sensor of the water potential Ψ. In the case of shortage of water (low Ψ) abscissic acid (ABA) is produced and affects the ion transport in such a way, that the stomata close. Finally there is a photo-active feedback RK_{pa}, which is controlled by a CO_2-sensor. The lower part of the figure shows, how environmental conditions influence CO_2-uptake, transpiration and the different feedback loops. After [1021]*

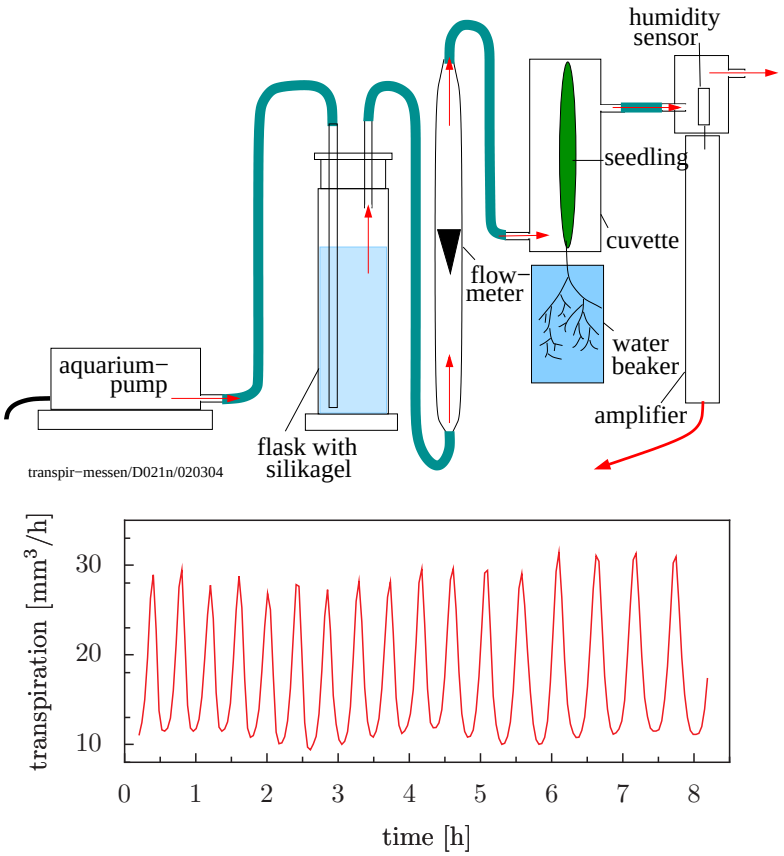

Figure 1.14: *Transpiration of primary leaf of oat. Top: Measuring device. Oat seedling with root system in water container. A pump presses dry air (dry silica gel in a washing bottle) through a flow meter over the primary leaf in a cuvette. The air is moistened by the transpiration and passes a humidity sensor (at the very right). This sensor produces a voltage (thick red arrow) which is a function of the humidity. It can be measured with a recorder or a computer. Below: Course of transpiration. Period length at* 27^0C *about 30 minutes*

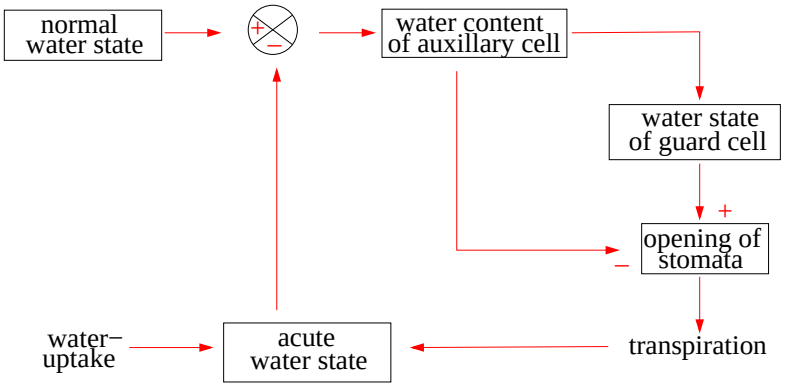

Figure 1.15: *Feedback-model of transpiratory oscillations after [713]: The normal water state is compared with the current water state. If the two values are different, the magnitude of deviation ('error') is transferred as a signal and leads -depending on the sign- to opening or closing of the stomata. In doing so, first the water content of the auxiliary cells and, with a certain lag, the water content of the guard cells is changed. The stomata determine transpiration, which together with the water uptake by the roots controls the water state of the plant*

responsible for the transpiration rhythm since oscillations are found also in oat with the root system cut off. A large resistance is, however, necessary. In the case of the oat it can be produced with the help of a clamp which compresses the xylem after cutting off the root system. Valinomycin can re-induce the transpiration rhythm in oats with a cut root system ([613]). This happens either because the resistance is increased, or because the substance increases the K^+-permeability.

There exist also *circadian* transpiration rhythms. They have been reported for a number of plants (reviewed by [61], [657] and [1534], see table 1.1). Rhythmic transpiration is probably more common than known so far. We found such rhythms in *Arabidopsis* and tobacco (unpublished. See also [1534]).

Table 1.1: *Plants with circadian control of transpiration*

plant species	conditions	period in hours	reference
Arabidopsis thaliana	LD and LL	24 resp. 23	[385]
Arabidopsis thaliana	LD and LL	24	[1534]
Avena sativa		26	[165]
Arachis	DD	26	[1128] [615]
Oxyria digyna	LL	24	[544]
Phaseolus vulgaris	LD,LL,DD	24, 25.7, damped	[657]
Phaseolus vulgaris	DD		[651]
Stellaria media	LL	23	[544]
Tamarix aphylla			[546]
Tradescantia virginiana	LL,DD	23.1,damped	[958]
Triticum aestivum			[986], [987]
Vicia faba	LL	22	[1409]
Vicia faba	DD	22	[514]
Xanthium pennsylvanicum	DD	24	[952]
Zea mays			[748]

1.6 Lateral leaflet movement of the automobile *Desmodium*

> *'Nature acts according to eternal, necessary, divine laws.....A phenomenon which appears to be brought about by intelligence, reason or just arbitrariness, astonishes or even frightens us.....But what a sensation if one observes* Hedysarum gyrans[2]*, which without external causes moves its leaflets up and down and by doing so seems to play with itself and with our comprehension. Everybody observing a banana plant exhibiting this ability to move its huge leaf parasols up and down should start back in horror´ ([1520])*

The circular movements of the lateral leaflets of *Desmodium gyrans* (*Fabaceae*) have given it its name 'Indian telegraph plant'. In India, one of its its native places, it is called Bon Charal ('forest churl', dancing to the clapping of the hands). The French name 'automobile' is less poetic, but as precise.

A leaf consists of a larger terminal leaflet and two lateral leaflets (one or both of the latter can be lacking). While the terminal leaflets show a circadian movement (horizontal during the day, hanging down during the night), the lateral leaflets move with periods in the minutes range rhythmically up and down continuously (figure 1.16)

The movements are based on volume changes of motor cells in special joints, the pulvini (figure 1.16). Their structure is shown in figure 1.17. A central cylinder contains the vessels and supporting elements. The motor cells surround the central cylinder. If the conducting vessels and supporting elements would lie at the periphery, as is normally the case in stems and leaf stalks, the organ could not bend.

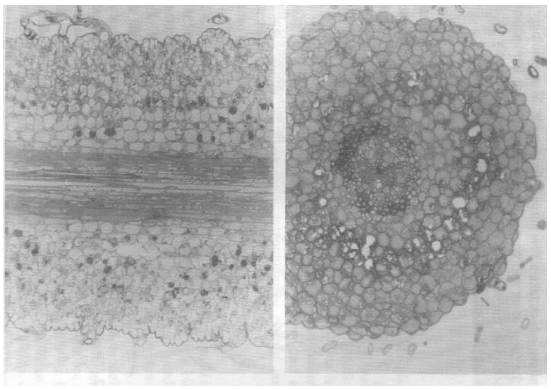

Figure 1.17: *Cross section (right) and longitudinal section (left) through the pulvinus of a lateral leaflet of* Desmodium gyrans. *From the periphery to the center: Epidermis, motor tissue, central cylinder. After [381]*

Bending and straightening of the joints is based on an alternating shrinking and swelling of the motor cells. In these cells cellulose-microfibrills are arranged in a ring like structure. It allows the motor cells to expand and shrink longitudinally, but a radial increase is prevented (left part of figure 1.18).

Electrophysiological studies and treatments with inhibitors have lead to the following model of the longitudinal changes in length of the motor cells (figure 1.18): Proton pumps in the plasmalemma pump H^+ out of the cell. In this way a negative charge is building up in the cell, which allows K^+ to enter the cell. Furthermore, via a H^+/Cl^- symport, Cl^- enters the cell. These osmotically active ions allow water to enter the cell: They swell. If the cell

[2] earlier name of *Desmodium gyrans*

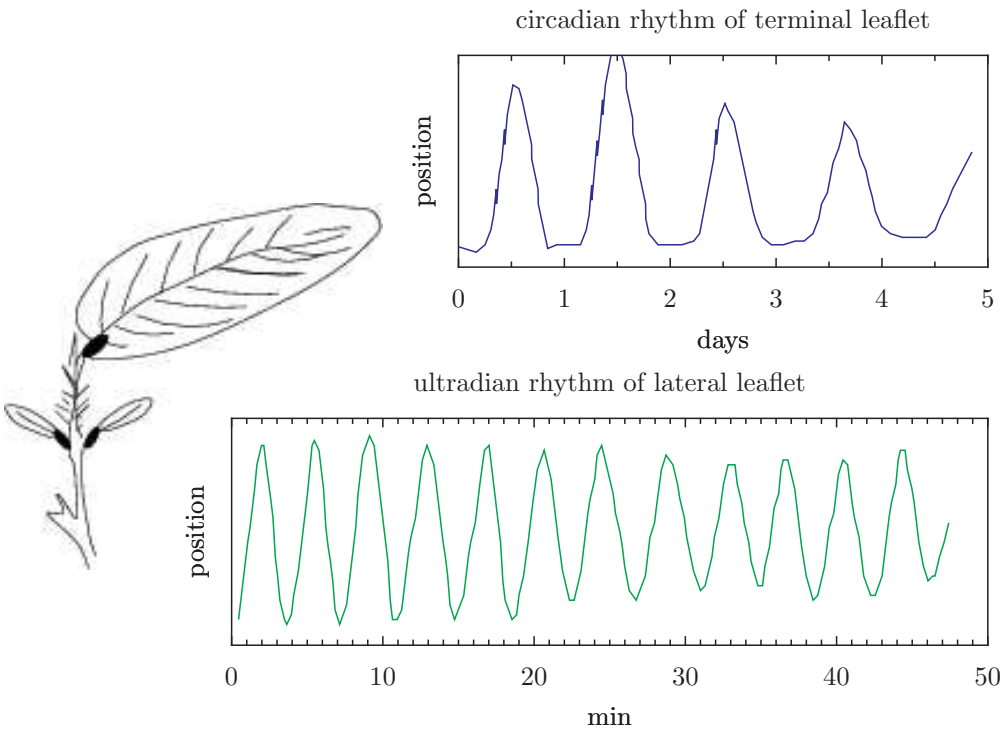

Figure 1.16: *Terminal leaflet (top) and lateral leaflets (below) of* Desmodium gyrans *and its rhythmic movement upward and downward. The upper curve shows the circadian movement of the terminal leaflets (x-axis: days). The lower curve presents the ultradian movement of a lateral leaflet (x-axis: minutes)*

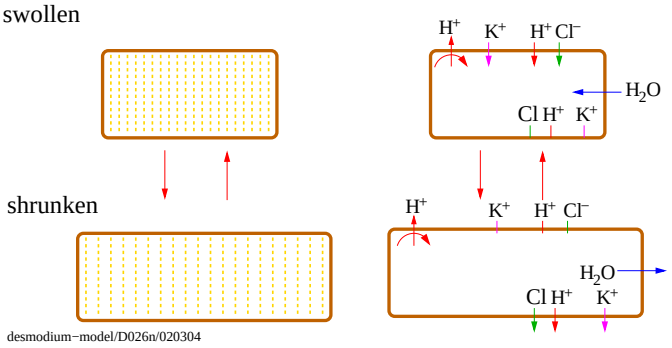

Figure 1.18: *Model of shrinking and swelling of motor cells in lateral leaflet joints of* Desmodium gyrans. *Left: Structure of cellulose mikrofibrils in a motor cell of the pulvinus of the lateral leaflet. Top in swollen, bottom in shrunken state. The longitudinal axis of the cell corresponds to the longitudinal axis of the join. Right: The shrunken motor cell (upper image), swells because it takes up K^+ and Cl^- and as a result water enters (driving force: Proton pumps, which expel H^+ from the cell to the outside. In this way a negative membrane potential is produced in the interior of the cell). Lower right part: The swollen motor cell shrinks, because the membrane potential breaks down and therefore K^+ and Cl^- leave the cell. As a consequence water flows out. After [381]*

wall is under a certain pressure, probably stretch activated Ca^{2+} channels open. Cl^- and K^+ flow out, water leaves the cell and it shrinks. Then the whole chain of events repeats itself: The proton pumps hyperpolarize the cell again. The rhythm and its period is determined by the time constants, which underlie these events. At higher temperatures the period length of the oscillation shortens.

Such rhythms in the minute range seem to be more common among plants than known up to now ([379]). They might have to do with the change in plant cells between a pump-state and the out-flow of ions from the cells interior ([519]).

1.7 REM-sleep of mammals

The examples of ultradian rhythms given so far are dealing with plants. Here is another example of an ultradian rhythm which is exhibited by mammals and birds: The REM-sleep. It will be treated briefly. Section 2.3 in the next chapter deals with the sleep-wake-cycle in man. A well done and detailed overview on sleep is presented as a CD by [127].

The sleep of humans is structured in different sleep stages. They consist of slow wave sleep stages (SWS) of varying depths[3] and of REM[4]-sleep. It takes about 30 to 45 minutes from the wake stage to reach the deepest stage of the SWS. Thereafter the process proceeds in the reverse direction: from the deepest stage of SWS to the flattest it takes another 30 to 45 minutes. However, one does not wake up. Instead the REM-sleep begins. This REM-sleep cycle is repeated every 90 to 120 minutes during the sleep and is thus an ultradian rhythm. It might be a part of the 'ba-

[3] Slow Wave Sleep 1-4
[4] REM= rapid eye movements

sic rest activity cycle' ([786]).

The REM sleep was discovered by [35], while studying the sleep of babies. They noticed rolling movements of the eye bulb at certain stages of the sleep under the closed lids of the eyes. While recording the brain currents in an electroencephalogram (EEG), regularities were found which are shown in figure 1.19. Every 60 minutes these 'rapid eye movements' occur in the baby. In adults the periods are longer. The following peculiarities are noticeable in this sleep stage:

- The EEG of the cortex is less synchronous as it is during the SWS, the amplitudes are lower, the pattern is similar to the wake condition.
- The EEG of the hippocampus however shows highly synchronized theta-waves (4-10Hz). These theta waves are also found during the waking stage, especially if the EEG of the neocortex is maximally desynchronized.
- The muscle tonus is low. Exceptions are the muscles of the eyes, the ear bone and the respiration.
- Homeostasis is lost. The body temperature tends to approach the temperature of the environment.
- Slow, rolling and occasionally saccadic eye movements occur ([308]). They are paralleled by electric activities in the brain stem, thalamus and visual and auditory cortex, so called PGO-spikes (pontingeniculate-occipital). They are the pacemaker of REM.
- Arousal threshold is high (insofar the REM-sleep is the deepest sleep), but spontaneous awakening is easy (insofar REM-sleep is the flattest sleep). 74-95% of the sleepers remember dreams during REM, whereas there are only 0-51% remembering dreams during the SWS (definition of dreams is different in the various studies, which explains the large variation of these figures).
- During the course of the sleep these changes between SWS- and REM-sleep are repeated 4 to 6 times. With time the REM-episodes become longer and the time between them shorter. Young adults spend about 25% of the sleep in REM, and half of it in SWS 2. The stages 3 and 4 are mainly found in the first half, the flatter stages and the longer REM stages in the second half of the night. Therefore awakening is usually in the morning.

A predator-prey-kind of model has been proposed by [639] which simulates the REM-sleep. The output of the FTG brain region[5] affects the *Locus coeruleus* (Lc) and feeds back to itself in a stimulating way with acetyl choline as the neurotransmitter. The Lc affects the FTG and inhibits itself via noradrenalin. However, later studies cast doubt on the validity of this model ([1376]).

For the biological significance of the REM-sleep see section 20.2 under 'special topics´.

[5] gigan to cellular field of the tegmentum, a pons region

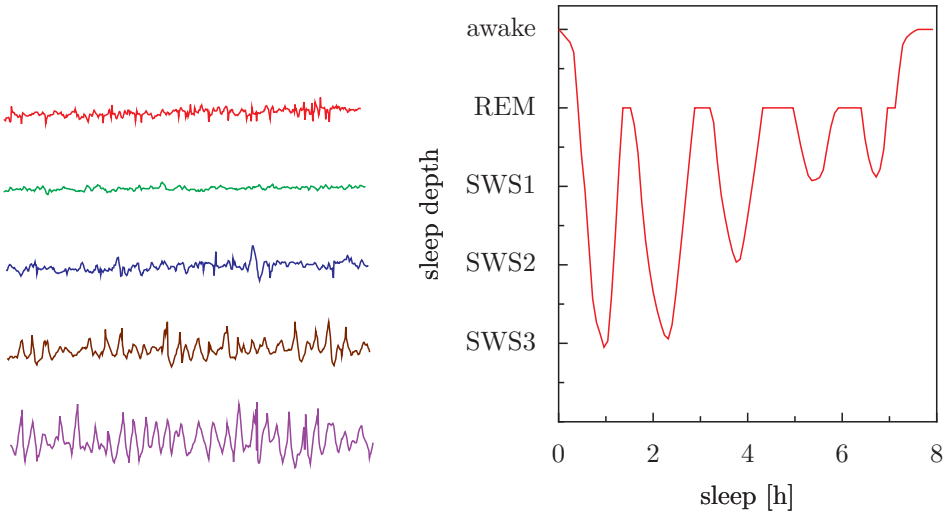

Figure 1.19: *Electroencephalogram-pattern of man in different sleep stages (SWS 1-4, REM-sleep) and during wakefulness (left part of figure, REM-sleep green, slow wave sleep SWS 1-3 blue, brown, purple, length of records 30 seconds each). Right figure shows the sequential occurrences of SWS and REM during the sleep (hours after onset of sleep). After [753]*

1.8 Ultradian and circadian rhythms

The relationship of ultradian and circadian rhythms is discussed by several authors (several contributions in [912]). According to [332] the former maintain circadian rhythms in *Drosophila*. On the other hand, circadian rhythms with different phase relationships to each other have been proposed to be the cause of ultradian rhythms. If the SCNs are leasioned, both ultradian and circadian rhythms disappear in hamster ([1581]). The properties of ultradian rhythms might change during ontogeny. Period length for instance can increase. The clarity of the ultradian rhythm correlates with that of the circadian rhythm. Ultradian rhythms seem to strengthen social synchronization in birds within the brood and flock members ([927]).

2 Rhythms in humans

Out of the numerous circadian rhythms of humans the sleep-wake-, activity- and body temperature-rhythm will be treated. Furthermore rhythms in the endocrine system are presented. Which models have been used to describe these rhythms? How is the circadian system of man organized and where are the controlling centers in the brain localized? Which role play circadian rhythms in shift work and jet lag? Finally some medical aspects are highlighted.

2.1 Introduction

In our body many rhythms occur from high frequency onces in metabolism, in the nervous system, in hormonal and excretory processes to very slow ones. The circadian rhythms are the best studied of all these rhythms ([1200]). What are their properties? How do they interact with other circadian, ultradian and infradian rhythms? Where are the controlling centers localized? How are they synchronized to the 24 hour day? What is the significance of these rhythms for the healthy person and the sick? To answer some of these questions will be the aim of the following sections.

We talked about ultradian rhythms in the preceding chapter. As an example of an ultradian rhythm in man the REM-sleep was presented. Infradian rhythms possess periods which are longer than daily rhythms. Monthly- and fortnight rhythms, annual rhythms[1] and rhythms with even longer periods belong to this group. In the next sections some circadian rhythms of humans are presented.

2.2 Circadian rhythms of humans

Adults are about two thirds of the day awake. The rest of the day they spend sleeping. This will remain so even without consulting man-made clocks. Our surrounding contains many informations concerning the time of day: Light and darkness, the position of the sun, traffic noise, the behavior of our fellow-man and of animals. These time cues ('Zeitgeber´ in German) can be used for the timing of wakefulness and sleep. But even without them our daily rhythm will continue. In rooms completely isolated from the environment and its influences, in caves, in the arctic or antarctic during the summer (continuous light) or during the winter (continuous darkness) the usual sleep-wake-pattern is kept. However, the 'subjective' day shifts away from the objective 24-hour day, because it is not exactly 24 hours but in most people longer. A person living in an isolated room without time cues could for instance possess a subjective day of 25.3 hours: Each day he or she would go to sleep (as an average) 1.3 hours later and wake up correspondingly later. After ten days her/his rhythm is shifted by 13 hours

[1] Annual rhythms have been found for conception, mortality, suicide rate, increase in length and weight in children, OH-corticosteroids, cortisone and testosterone ([32], [1245], [1246])

in respect to the outer world (figure 2.1) This demonstrates that the sleep-wake-cycle of man is controlled by an internal clock, which is circadian (period length of *about* 24 hours). It is normally synchronized to the 24 hour day by Zeitgeber of the environment. Without these time cues the internal clock shows free run.

Even in a normal day one can observe occasionally in some humans free run (figure 2.2).[2] Some babies might show it before they become synchronized to the 24-hour-day (left part of figure 2.3). Their sleep-wake pattern is not as clear as the one in adults, because it is superimposed by an ultradian rhythm of about 4 hours. It is however recognizable in figure 2.3 from week eight until week sixteen and exhibits a 25 hour period. After that time the synchronization to the 24-hour-day is established. The example on the right side of the figure demonstrates in an Indian baby, that the daily rhythm can be synchronized already from birth onward. See also the special topic 'Ontogeny of the circadian system in man' (section 20.3).

This free run under normal conditions is often observed in blind persons (see section 20.5).[3]

Besides sleep/wakefulness and locomotor activity, the core and skin temperature are also under circadian control. Circadian are furthermore cardiovascular and respiratory, metabolic and gastrointestinal

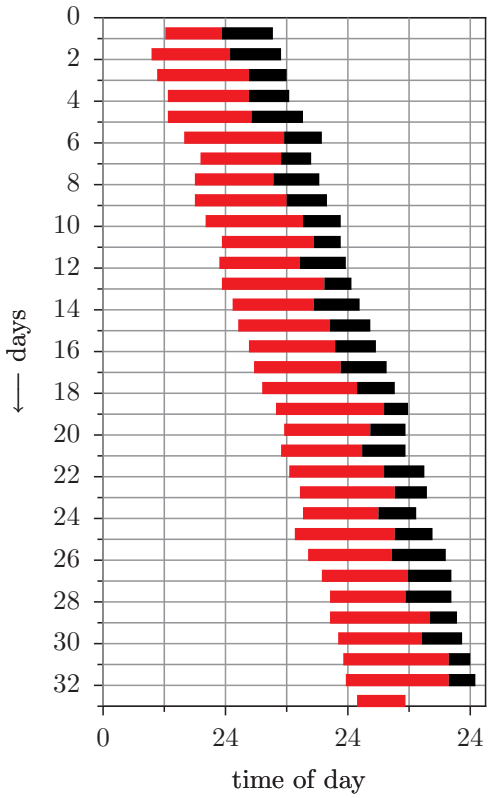

Figure 2.1: *Sleep-wake-rhythm of a subject in a subterranean apartment without Zeitgeber information. The daily wake times (red bars) and sleep times (black bars) are shown for 32 days underneath each other. For a better overview three successive days are plotted on the x-axis. However only one wake- and sleep time are shown in each row. After [1556]*

[2] Thus [802] reported the case of a 34 year old, who exhibited for 8 years a 24,8 hour rhythm of his sleep-wake-pattern. The author presumed a physiological anomaly in the regulation of the circadian system or in the synchronizing mechanism. We ([499]) studied the circadian behavior of a student, who showed a free run in spite of living in a normal environment.

[3] [1008] studied 50 blind persons. The sleep-wake-rhythm of 20 of them was disturbed. One of them free ran with a period of 24.9 hours.

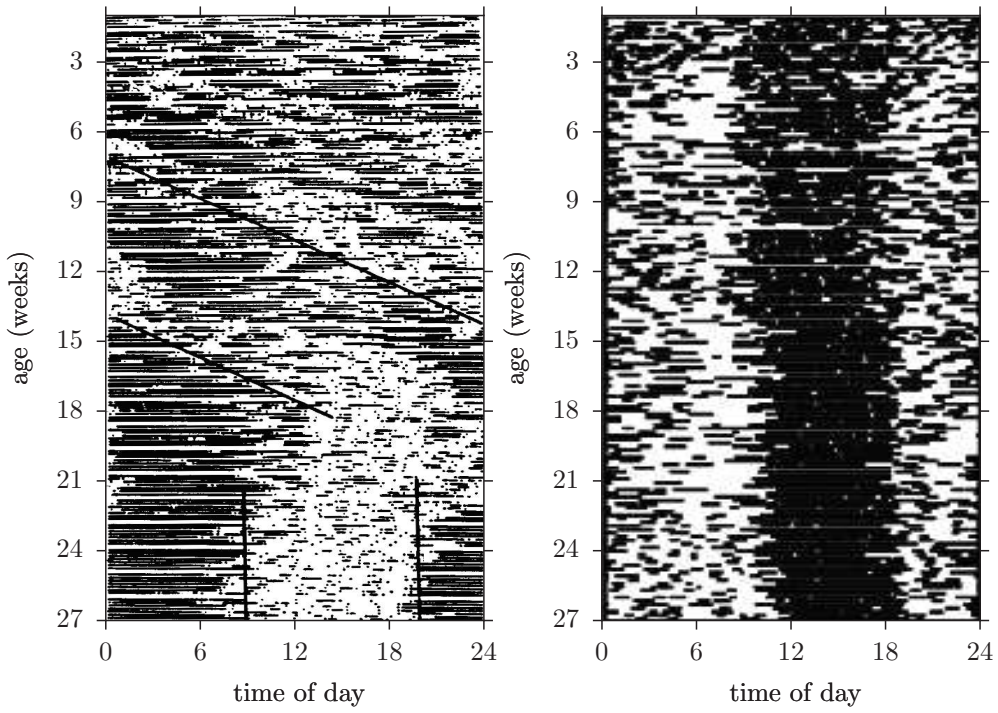

Figure 2.3: *Left: Drinking- (dots), sleep- (horizontal broken line) and wake times (bright bars) of an american baby in the first 26 weeks after birth. Between the eighth and the seventeenth week a free run with a period length of about 25 hours is recognizable (inclined lines). From the nineteenth to the twenty first week the sleep-wake-pattern of the child is synchronized to the 24-hour day (vertical lines in the lower part of the figure). After [788]. Right: Slep (horizontal lines) and wake time (bright bars) of a south-Indian baby druing the first 184 days after birth. The sleep-wake-pattern is synchronized from the very begin to the 24-hour day, Data from Marimuthu, Madurai, Tamil-Nadu, India*

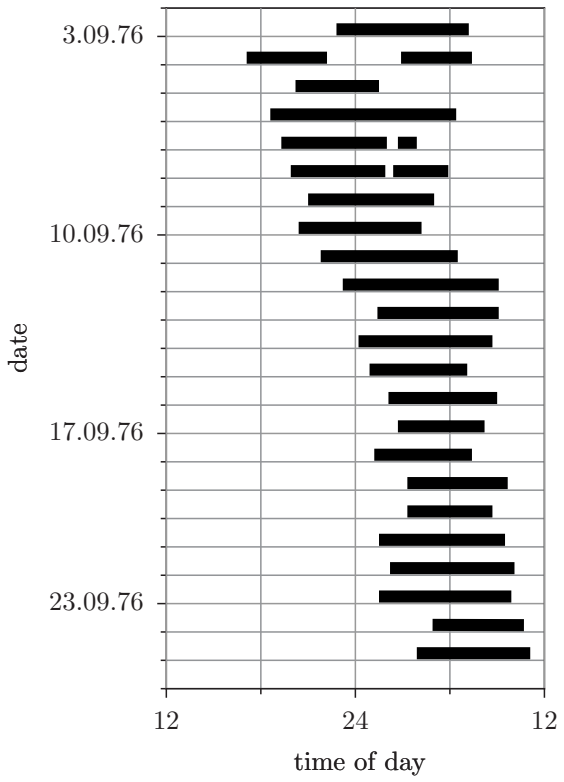

Figure 2.2: *Free run of sleep-wake rhythm of a person in spite of normal environmental conditions. Successive days shown on y-axis, time of day on x-axis. During dark parts the person was asleep, during the part indicated by a line awake. Note partial synchronization during September 22 to 29. After [499]*

rhythms. The kidney and hormones affecting it, mental performance ([243]), the endocrine system and many other processes in the body of man are controlled in a circadian way ([1013]). Before talking about some of these rhythms, we should consider the characteristic properties of circadian rhythms. See also [1013], [1029].

2.2.1 Properties of circadian rhythms

Circadian rhythms show a number of characteristic properties:

1. They are *self-exciting oscillators*. That is, they show circadian rhythms (‘*free run*´) even under conditions of constant environmental conditions: The period length is as a rule somewhat shorter or longer than 24 hours (see page 395).

2. Circadian rhythms can be *synchronized*. Zeitgeber are mainly the light-dark-cycle of the day, but also daily temperature differences, electromagnetic fields. In man social time cues and man made clocks are also synchronizing.

3. The period length of circadian rhythms is hardly affected by the average temperature of the surrounding. Instead it is ‘*temperature compensated*’ ([174], see also section 18.5).[4]

[4] There are regions without temperature deviations such as some tropical regions or tropical seas. The period length of tropical plants is indeed stronger dependent on environmental temperature as it is in other plants. *Phaseolus mungo* for instance has at 17^0C a period length of 32 hours ([973]). In *Neurospora* there is a mutant, in which the temperature compensation of the conidiation rhythm has been lost ([922]). The temper-

4. The period length of circadian rhythms can be influenced by different factors and conditions. Examples in man are: Light intensity, light modality, electromagnetic fields, work load, psychological factors, social contact ([1556], [1029]).

5. Circadian rhythms are already found in *unicellulars* and in *prokaryotes* (see chapters 4,5,6).

6. Circadian rhythms are genetically programmed. This was already expected by [290] and experimentally shown to be so by [179]). Today this property is extensively used to try to unravel the underlying mechanisms with the help of genetic methods and by using mutants.

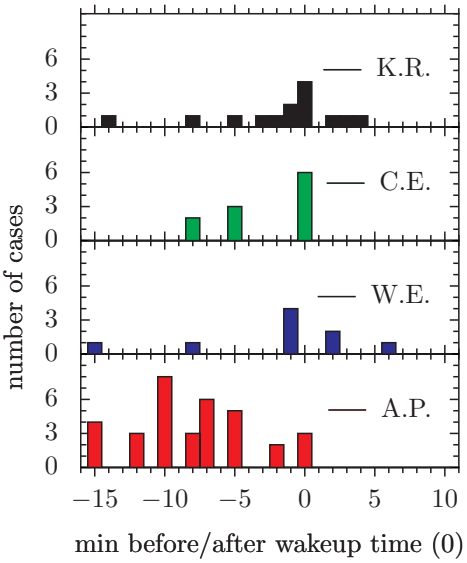

Figure 2.4: *Some people possess a head clock. It enables them to awake at night times without an alarm clock. Here are four examples given of persons who were able to use their head clock quite efficiently. After [236]*

2.2.2 Why are circadian rhythms important for man?

Circadian rhythms help man to adjust to the time structure of the environment.

For instance a certain *time point* can be important and triggered or remembered by the circadian system. The alarm clock of humans (head clock) might belong here: Some people are able to wake up at a distinct time during the night. For instance they, before falling asleep, decide to wake up at 3 o'clock in the night if they plan so in advance. This they manage with a surprisingly high precision (figure 2.4). It has, however, not yet been shown that this head clock uses the circadian clock. The 'Zeitsinn´ (sense of time) of insects is an animal example (see page 170).

On the other hand metabolic and physiological processes, mental performances or ways of behaving are adapted to the daily rhythm (or also to the monthly or annual rhythm) of the external world. In this way *ordered patterns of time* are formed which adjust metabolism, physiological events, development and reproduction to the environment. In the case of photoperiodic events ([1469]) a circadian rhythm is used, which measures day-length and determines in this way the season. Photoperiodic time measurement is the most important Zeitgeber for annual rhythms. Whether in humans *photoperiodic reactions* exist is discussed (see page 304). There are, however, indications that *annual rhythms* exist in man ([32], [1245], [1246], see page

ature compensation is thus not necessarily an essential property of the circadian mechanism. On the other hand there are ultradian rhythms which possess temperature compensation (see page 17 and [912]). Furthermore, the type of temperature compensation might depend on the metabolic situation, as shown in *Euglena* ([155]).

304). Circadian rhythms are used in animals also for orientation, as for instance in sun compass orientation (see chapter 10).

2.3 Sleep-wake rhythm in man

Each of us spends about one third of the day in sleeping. This amounts in a 60 year old to 20 years. In spite of its importance and in spite of numerous studies, sleep is still not well understood. Questions such as 'why do we sleep?' or 'what is sleep?' (see special topic 20.7) are difficult to answer, because we do not know enough about it. However, progress is made ([1087]).

2.3.1 Why do we sleep?

Why-questions are somewhat dangerous in biology being a scientific field: They implicate a purpose (teleonomy) which in reality does not exist. Somebody once stated

> 'Teleonomy is like a woman whom one loves very much but with whom one can't show up in the public'.

However, sleep must be essential. Otherwise it would have been eliminated by selection. All mammals spend a significant portion of its life sleeping ([198]). Longer sleep deprivation are harmful or even fatal ([413]).

The cause of sleep is a complex question ([1087]). Its phyllogenetic appearance in mammals and birds is probably closely connected with the evolution of their brains from an anterior brain to a multi-layered neocortex (mammals) or neostriate (birds). It had to cope with two different waking types. The older one developed for diurnal activity in the light. In mammals this early waking type which was controlled by the brain stem, was suppressed (and survives as *inactivity* in the slow wave sleep). Waking was displaced to the cortex after homeothermy and nocturnal life took over. The nocturnal rest of poikilotherms might have resulted in rapid eye movement sleep. The complex structure of mammalian sleep might thus be a remnant of evolution. Cortical control of waking was the true acquisition in mammals.

A number of proposals were discussed, why we sleep:

- Sleep is the consequence of being awake. If we or an animal are a certain time of the day active, we would automatically become tired and fall asleep. This was tested experimentally. Rats which are kept in a cage with access to a running wheel and which normally run up to seven km per night were suddenly denied from using the wheels. In spite of it sleep occurred as usual. The activity during the wake time is thus not the cause of sleep. On the other hand, the propensity to sleep is increased by the duration of the wake time.

- Sleep reduces expending of energy. Sleep would according to this proposal be a saving measure, which avoids exhaustion. However, the forsaid applies here too.

- Sleep is a means of recovering. During sleep the central nervous system could be maintained and repaired.

- It has also been suggested that we sleep in order to avoid dangerous sit-

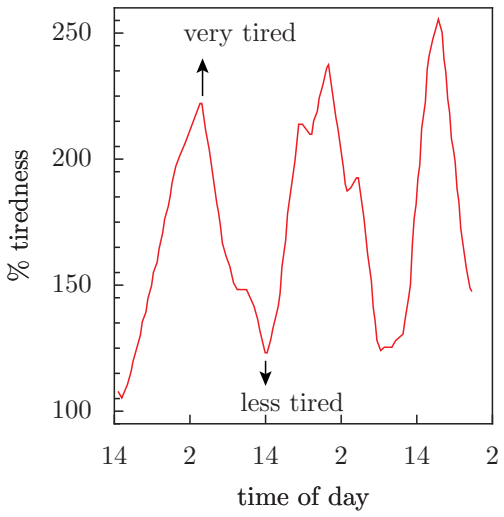

Figure 2.5: *Circadian rhythm of tiredness during sleep deprivation. Fifteen female subjects were kept awake for 72 hours. They graded their tiredness every three hours (average values on a scale with 100% as the normal tiredness). After [5]*

uations during the night such as darkness, low temperatures, predators.

- Do we fall asleep, because it is time to sleep? In favor of this proposal is the observation, that in the morning from a critical time point onward the tiredness disappears again if we did not sleep during the night (figure 2.5).

Tiredness fluctuates apparently in a daily rhythmic way. This suggests that an internal clock controls sleep. We come back to it again (see subsection 2.3.3).

2.3.2 Physiology of sleep

Sleep is investigated by recording the electric potentials from the scalp (EEG) or from the surface of the cortex (ECoG). The signals can be processed yielding a quantitative measure of the different frequency components ([656]).

The neurophysiological basis of the EEG signals are studied intensively and progressed considerably ([918], [1420], [1421]). The dominant frequencies in the slow-wave sleep EEG (slow waves and spindle oscillations) correlate with oscillations in thalamocortical neurons. If the brain changes from the waking or the REM state (which are desynchronized) to the slow-wave sleep (which is synchronized) the membrane potentials of the thalamocortical neurons become hyperpolarized. Furthermore these neurons start to fire in rhythmic bursts. Calcium influx and sodium mediated action potentials are involved. These events correspond to the EEG observations ([3]).

Recent experiments have shown ([470]) that a certain sleep promoting cell population in the ventrolateral preoptic nucleus (VLPO) of the preoptic area have a sleep-promoting function. It is a homogeneous group of cells, which are inhibited by monoaminergic and cholinergic neurons of arousal systems during the wakefulness state. They are therefore inactive during wakefulness. At sleep onset these neurons increase their firing rate under the influence of circadian inputs from the retina and the SCN and under the influence of homeostatic factors (body temperature) and sleep promoting factors. The increased activity of their GABAergic neurons inhibits the arousal centers to which they project. This leads to dis-inhibition, thereby further increasing their activity. All this facilitates the ability of these neurons to promote sleep (see figure 2.6 and [470]).

There might be cell groups with similar characteristics in other regions of the basal fore-brain/preoptic area. But retinal and

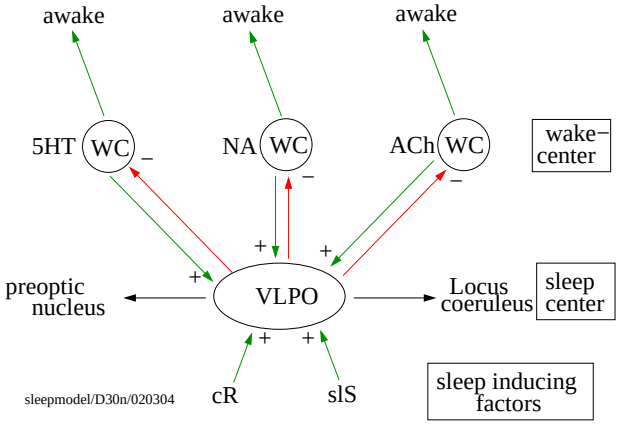

Figure 2.6: *In an so far unknown way the circadian system (cR) and sleep substances (slS) of the body stimulate the 'LTS' (low threshold spike-cells) in the VLPO of the preoptic area of the brain. This leads to an inhibition (-) of wake centers (WC) via GABAergic neurons (red arrows upward). These wake centers induce wakefulness via serotonergic (5HT), noradrenergic (NA) and cholinergic (ACh) neurons (greeen arrows at top). The wake centers (WC) de-inhibit the VLPO area (green arrows, +), thus increasing the inhibition of the wake centers (WC) by sleep-promoting GABAergic LTS-neurons further. After [470]*

SCN inputs are found in this ventrolateral preoptic nucleus area only.

The genetic and molecular control of sleep is also studied by searching for sleep genes in mammals ([803]).

2.3.3 Circadian control of sleep

That the sleep-wake-rhythm of man (and of other mammals) is under the control of a circadian clock becomes evident under constant conditions without time cues. The sleep-wake cycle will continue, but with a period which in most people is longer than 24 hours. It is as an average 25 hours long (range from 23 to 28 hours). The control of sleep by a circadian oscillator is furthermore shown by a limited range of entrainment: If we try to force us or others to live in a non 24-hour day, we will succeed only to certain limits (range of entrainment is 18.5-33.5 hours, see page 58). SCN lesions in squirrel monkeys have shown that sleep is actively promoted or facilitated by the SCN. Homeostatic processes control sleep in addition to the circadian control by the SCN ([357]).

The oscillator controls also the locomotor activity (figure 2.1) and other events. However, a number of findings indicate, that the control of the whole circadian system is not exerted by just *one* circadian clock. Apparently, a multioscillatory system is involved, as discussed later (subsection 3.5).

For literature on the circadian control of sleep and temporal characteristics of sleep see [1535].

2.3.4 Sleep disturbances and circadian rhythms

Frequently sleep disturbances occur[5], which can be quite annoying or even dan-

[5] About 15% of the population in industrialized countries complain about chronic, and 20%

gerous. Narcolepsy is an example,[6] sleep walking another. Sudden infant death syndrome (SIDS) occurs during sleep. For more details on sleep disturbances and therapies see [350], [1231] and [752].

Sleep disturbances are classified into

1. initiating and maintaining sleep,
2. insomnia,
3. hypersomnia (e.g. narcolepsy, sleep-apnoe, idiopathic hypersomnia),
4. disorders of the sleep-wake schedule and behavioral dysfunctions associated with sleep (e.g., especially in children, night mares, sleep walking, enuresis).

We will restrict the discussion to sleep disturbances connected with the circadian system.

Temporary insomnia is often the result of changed phase relationship of the sleep in respect to the day-night-rhythm. This relationship can be disturbed in shift workers (see section 2.10) and due to jet lag (see section 2.11). In the case of delayed sleep phase syndrome, falling asleep is disturbed. It occurs often in persons belonging to the evening type. They do not have problems if they can sleep for instance between 4:00 and 12:00 o´clock. That is, their sleep per se is not disturbed. It is only wrongly adjusted to the day-night rhythm. In such cases a therapy is adequate in which the rhythm is readjusted by 'reset´. Sleep disturbances of many shift workers could be cured by using better shift schedules, as discussed in section 2.10. Even the 'normal´ sleep pattern might not be optimal.

There are indications for a bimodal sleep (siesta in addition to the night sleep) being more natural (figure 2.7, [1542]) and more relaxing ([1430]). It is more pronounced in the elderly subjects ([203]).

In some cases the sleep time of a person is not synchronized to the 24 hour day. Instead those persons show free run with a period length according to the speed of the circadian clock and they have difficulties during periods where their active phase coincides with the normal night time ([802]). The sleep of blind persons is often disturbed for the same reason (see section 20.5).

Sleep disturbances are found in a number of mental diseases ([1270]) such as schizophrenia ([1261], [300], [757]), epilepsy ([69]) and endogenous depressions ([1403], [1262], [505]; see also section 20.11). In narcoleptics ([728]) the circadian system is changed, but principally intact. Schizophrenics go early to bed and rise early. Manic depressives go to bed late and rise late. Apparently in narcoleptics the ultradian rhythm is abnormal (fragmented) and extends into the waking state ([1212]). With a short sleep during the day (nap) the narcoleptic attacks can usually be avoided ([1050]).

about occasional sleep disturbances. In the Unites States about 29 to 39% of the inhabitants older than 18 years suffer under it (that is 45 to 60 million people). 8 to 12 million of them are under medical treatment, 4 to 6 million use sleeping pills. In 1977 about 25.6 million sleeping pills were prescribed, and another 30 million which were bought without prescription.

[6] Sudden short sleep attacks during the wake time. They are connected with low muscle tonus ('cataplexy´) and last usually for 10-20min. An empty glance is typical. One is dealing with a fragmented REM-sleep; the EEG is REM-sleep-like. After the attack the person feels refreshed. Routine work is continued, but with many errors. Because of the atonia this condition might lead to car accidents. In about 0.04 to 0.09 % of the cases of narcolepsy the affected persons fall into coma.

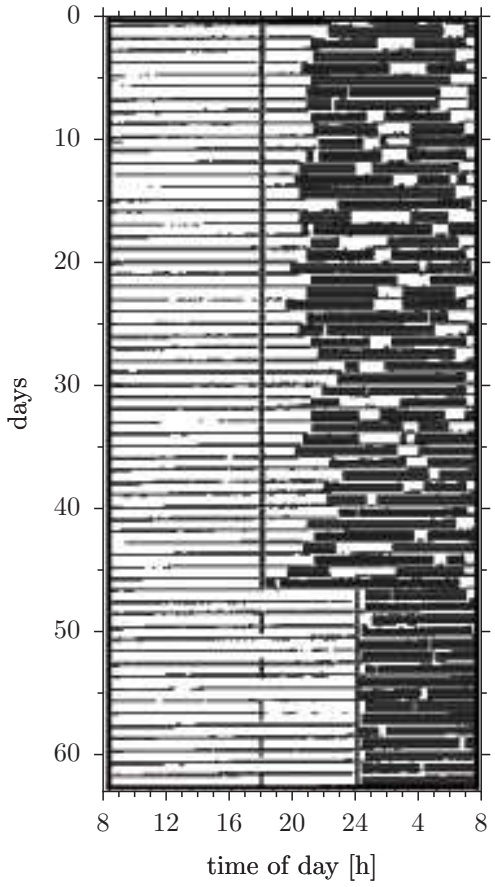

Figure 2.7: *Sleep of a young woman under long artificial 14 hour nights, upper part, and under 8 hour nights (bottom). The black bars show electrophysiologically monitored sleep. Note the bimodal sleep pattern in the long nights with 3-5 hour sleep bouts separated by 1-2 hours of wakefulness. This might have been the normal sleep pattern before the industrial revolution where many people slept from sunset to sunrise. After [1542]*

2.3.5 Models of the sleep-wake cycle

Models are used to describe complicated systems in a simplified way. They are testable by using the models for predictions and checking these experimentally. If the predictions are not met by the model, it has to be changed and tested anew.

This shall be illustrated using models for the sleep-wake cycle as examples: In testing, whether a model is correct, experimental results are accumulated and compared with the predictions. For example, the duration of sleep as a function of onset of sleep, bimodal onset of sleep, a six hour forbidden zone of waking up before the body temperature minimum, the occurrence of a point of singularity can be used for tests ([698]).

The following models were specifically designed for explaining the sleep-wake cycle:

- The two-process-model of sleep regulation by [284] (see also [283]). A circadian rhythm C (sleep-independent) and a homeostatic process S (sleep-dependent) interact mutually. A threshold H for falling asleep and a threshold L for waking up are important (see figure 2.8). The model was tested in rats and man.

- [1573] explains sleep duration as a function of the time at which one falls asleep with a topological model.

- A thermoregulatory model of sleep control in humans was described by [1053].

Other models are mentioned later (page 53).

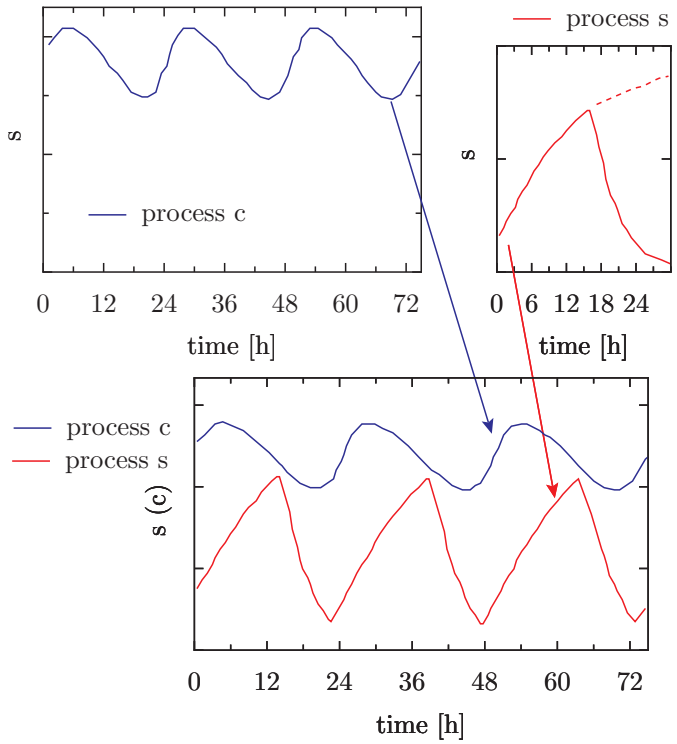

Figure 2.8: *A two-process-model of sleep regulation: A circadian rhythm (process c, sleep-independent, blue curve) and a homeostatic process s (sleep-dependent, red curve) interact mutually. A threshold for falling asleep (when the red curve touches the blue one) and for waking up (red curve reaches a threshold at its lowest point) are important. The model was tested in rats and man. After [284], see also [283]*

2.4 Circadian control of body temperature

The mechanism of temperature control in homeothermic animals is reviewed on page 421. The body temperature is not constant, but oscillates diurnally. This rhythm is endogenous, since it is found also under constant conditions independent on activities (figure 2.9). It is controlled by an oscillator which determines also the composition of the urine, the cortisol secretion and other events.

The daily variations in body temperature were already observed by [298]. [1387] described temperature-cycles of a rhesus monkey not only in a light-dark cycle, but also under continuous light. This demonstrated the endogenous nature of the body temperature fluctuations (known already much earlier with other functions of the human body). [994] studied circadian fluctuations in the body temperature of bats. [1198] found a mutant of the hamster, the body temperature of which fluctuated in cycles of 20 instead of 24 hours; during the course of 5 days it thus underwent 6 circadian cycles. Between 1967 and 1990 about 2700 publications appeared on circadian rhythms in body temperature, that is more than 100 per year. A more recent article by [1211] gives an overview.

The heat *production* participates with 25% in the rhythmic fluctuations of the body temperature, whereas the heat *loss* (skin, blood circulation, badly isolated body parts such as the extremities) with 75% (the values depend on the environmental temperature). *Heat loss* is thus especially strongly under circadian regulation. The body- and skin temperature rhythms differ in their phase relationship to each other (see figure 2.10). Changes in

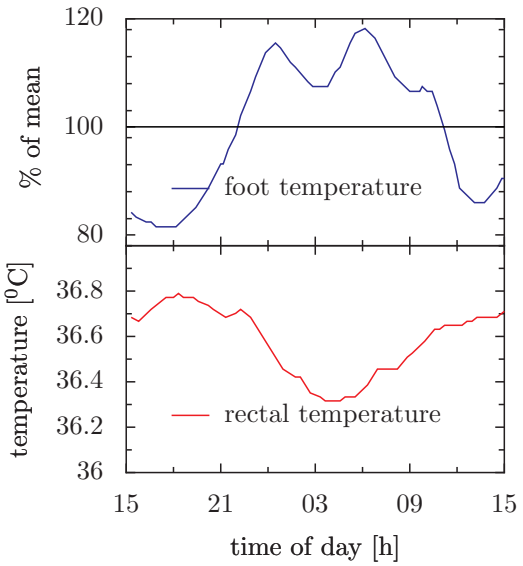

Figure 2.10: *Deep-body- (core) and foot-temperature-rhythms are out of phase with each other: Whereas the core temperature (measured rectally, in degrees Celsius) is higher during the day as compared to the night, the foot temperature is higher in the night (deviations from the daily mean, in percent). After [636]*

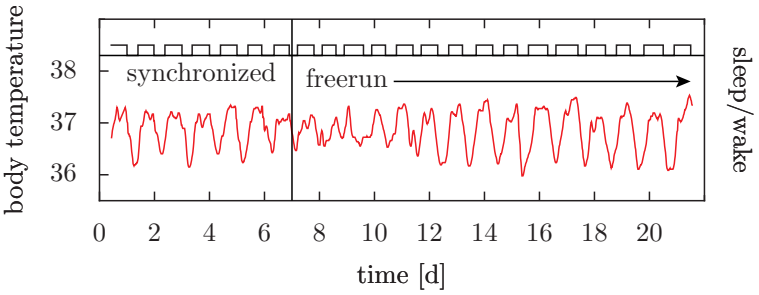

Figure 2.9: *Course of body temperature in man in synchronization with the 24-hour day (first part, 'synchronized') and under free run (second part, 'free run'). Top row shows periods of wake (white) and sleep (dark). After [33]*

blood circulation to and from the skin of the extremities, variations in the size of the central reference value and how sensitive or how effective thermoregulatory effector mechanisms are play a role in this game. Furthermore the behavior feeds back to it.

The body temperature of a person which sleeps from 23:00 to 7:00, changes typically (inhabitants of Paris and Sydney) in the following way: 3 hours before waking up 36.5^0, at 9:00 37.2^0, at 20:00 37.4^0, and at 4:00 36.5^0. The maximum is reached at 17:00. In inhabitants of Colombo (Sri Lanka), however, the maximum is reached already at 12:00, although they rise up and go to bed just one hour earlier. The reason for this difference is unknown.

Under constant conditions and without Zeitgeber the period length is around 25 hours as an average. In contrast to entrained conditions the body temperature rhythm is by 4 hours advanced in respect to the activity rhythm[7].

Sleep occurs under constant conditions at times of low body temperature. Naps, however, are taken at times of high body temperature.

Most studies on body temperature-rhythms were performed at neutral environmental temperature (32^0C), at which thermoregulatory reactions must hardly be activated. In some cases, however, the environmental temperature was also varied. It turned out that at higher temperatures of the environment the amplitude of the rhythm decreased, at lower temperatures it increased (Squirrel monkeys: by 1.6^0 at 32^0, by 2.4^0 at 20^0 and by 1.0^0 at 36^0). The reason for this difference is, that the heat loss is under strong circadian control, and at lower environmental temperatures heat loss is more pronounced.

The body temperature changes often in a biphasic way, shown by a dip during noon time (figure 2.11, [31]).

Why are there rhythms of the body temperature at all? The energy saving is, at least in larger mammals, too low (namely 260 kJ per day at 1^0C amplitude). That is less than 3% of the 2400 kCal per day. In addition, this difference in temperature during the course of a day is also present during hibernation (see subsection 12.4.4). The question can not be answered so far.

[7] but not in animals: here the circadian rhythm of the body temperature damps out in conditions without Zeitgeber. Activity rhythm and drinking rhythm are also damped. Perhaps under constant conditions desynchrony builds up which is responsible for the damping. The rhythm can be re-initiated again.

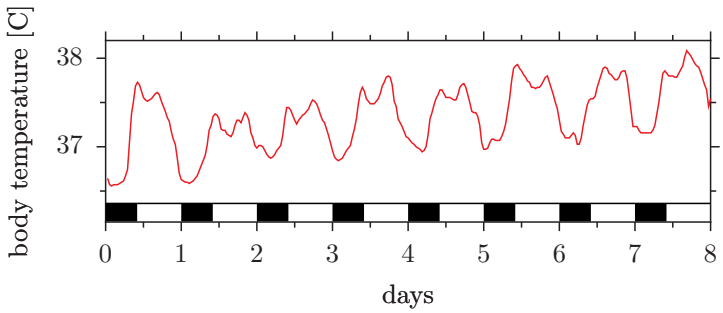

Figure 2.11: *Biphasic course of body temperature in a women, with somewhat reduced temperature around noon time. Increase in the average mean body temperature caused by the estrus cycle. After Zimmerman in [1029]*

Besides circadian oscillations there are also infradian ones (the menstrual cycle in the female: 0.4^0C higher at the onset of the luteal phase after ovulation) and ultradian (0.3^0C cycle of the axle-temperature with a period of 30 minutes, 2-6 hour rhythms of the abdominal skin temperature in newborns).

Body temperature and sleep-wake cycle. Normally the body temperature- and the sleep-wake cycle are in synchrony with each other. Likewise the circadian rhythm of activity and of body temperature run parallel. At the time of maximal body temperature activity is also high. Under certain conditions, however, they are not coupled with each other anymore and can depart. An internal desynchronization has taken place (figure 2.12). From this it was concluded that several oscillators are hierarchically and non-hierarchically coupled with each other.

There are further indications, that the circadian rhythm of the body temperature is not merely the automatic consequence of rest and activity:

1. The circadian rhythm of body temperature is earlier as that of activity, that is, temperature has already increased before rising from bed.
2. Although the body temperature decreases at the time of sleep onset, this amounts only to 10% of the value it fluctuates during the whole temperature cycle.
3. At a bed rest throughout the day the expression of the circadian rhythm of body temperature (amplitude) is only slightly lower as usual. The same is true, if no food has been taken for 24 hours.
4. During sleep deprivation for several days the circadian rhythm of body temperature is pertained.
5. Morning types and evening types possess a very similar body temperature rhythm, although the activity rhythm differs.
6. Under continuous activity and food taken up equally spaced over the day and night the circadian rhythm of body temperature continues, only amplitude is somewhat reduced.

Apparently body temperature and activity are driven by separate circadian oscillator.

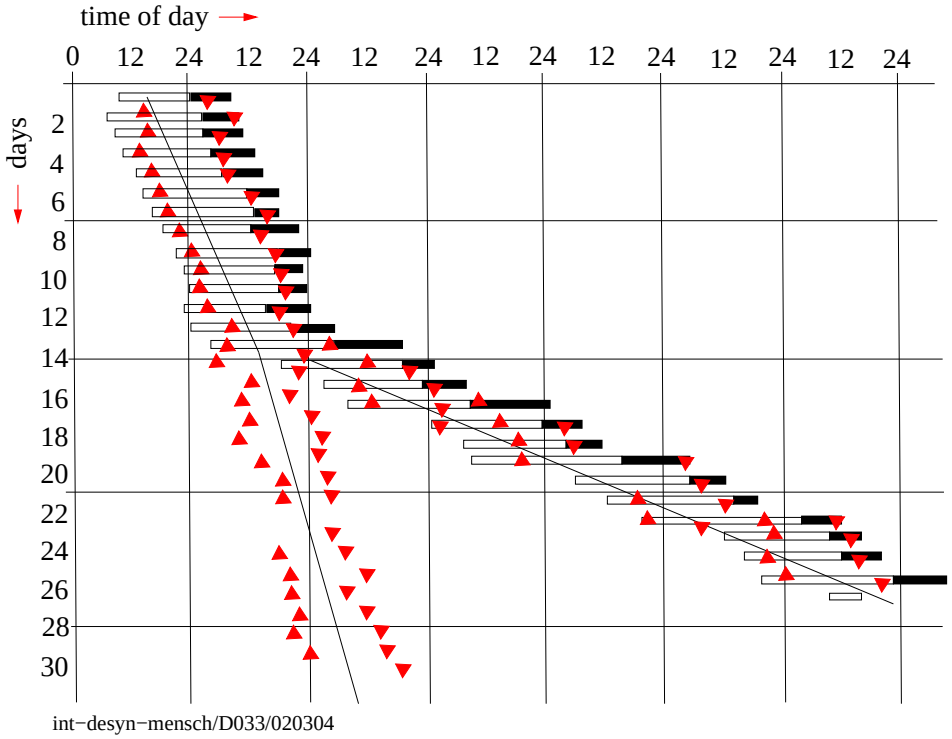

Figure 2.12: *Internal desynchronization in man between body temperature- and sleep-wake rhythm under Zeitgeber-free conditions in an subterranean apartment: In the first fourteen days the daily maxima and minima of the body temperature run parallel to the sleep-wake cycle. The period length is for both events 25.7 hours. Afterward the body temperature rhythm shortens somewhat (period length 25.2 hours) and the period of the sleep-wake-cycle increases considerably to 33.4 hours. In order to see the course better, the days were not only plotted beneath each other, but in addition seven days side by side. Therefore the values and curves should show up seven times next to each other. However this would blur the impression and was therefore omitted. After [1556]*

Does the circadian control of body temperature affect the reference value of the temperature, with other words, is the reference value changed in a circadian way? A number of findings speaks in favor of it ([1211]). For instance, the body temperature fluctuates in a circadian way also, if a person sleeps very long (for instance if sick). Fever increases the reference value of the temperature by increasing the heat production and decreasing the heat loss. However the body is much more complicated as compared to an ordinary thermostat made by engineers. It lacks a static reference value. Instead, the reference value is influenced by internal and external factors.

Alternatively the circadian rhythm of body temperature could easily be brought about by a rhythmic control of heat production and heat loss and not necessarily by a reference value changed by the circadian system ([1241]).

2.5 Rhythms in the endocrine system and during reproduction

The endocrine system of man is also under circadian control. Some hormones are, however, additionally secreted in an ultradian way as, for instance, the sexual hormones LH and FSH.

Figure 2.13 shows a scheme of the diurnal control of hormones in the example of cortisol and conjugated corticosteroids. Cortisol and conjugated corticosteroids are the most important corticosteroids of mammals. Corticosteroids are made from cholesterol in the adrenal gland. The cortisol-concentration is high in the morning and low in the evening. A minimum is reached in the first two hours of sleep. Maximal concentrations are found at the time of waking up. Afterward the cortisol concentration declines again until 1-2 hours before sleep onset. If with a catheter every 20 minutes blood samples are taken and checked for the amount of corticosteroids, 6-9 episodes per day are found, i.e. an ultradian rhythm which superimposes the circadian rhythm (see figure 2.14 and chapter 1). The rhythm is neither influenced by the behavior nor by rhythmic changes in the environment. Otherwise it would not occur under constant bed rest, at sleep deprivation and under regular and uniform food supply. In newborns this rhythm is lacking. It develops at an age of 2-3 years.

If Zeitgeber are absent, the cortisol rhythm in the plasma has a period length of 25 hours. The period is not changing under internal desynchronization. The most important Zeitgeber of this rhythm is the light-dark-cycle. In people which were already blind at birth the cortisol rhythm shows occasionally free run. Stress increases the cortisol concentration. This masks the rhythm. In rats it was demonstrated that corticosteroids are secreted also in vitro rhythmically ([1119]). It is unknown where the rhythmic control occurs.

Other hormones such as vasopressin and oxytocin are likewise secreted in pulses every 60 to 120 minutes (see chapter 1). Afterward they are metabolized. The half life time is in the range of minutes to hours. The episodic course makes the determination difficult and rather variable if the sampling rate is too low (every 3-4 hours).

The amplitude of the growth hormone GH is high and fluctuates around 100%. The maximum occurs during the first two

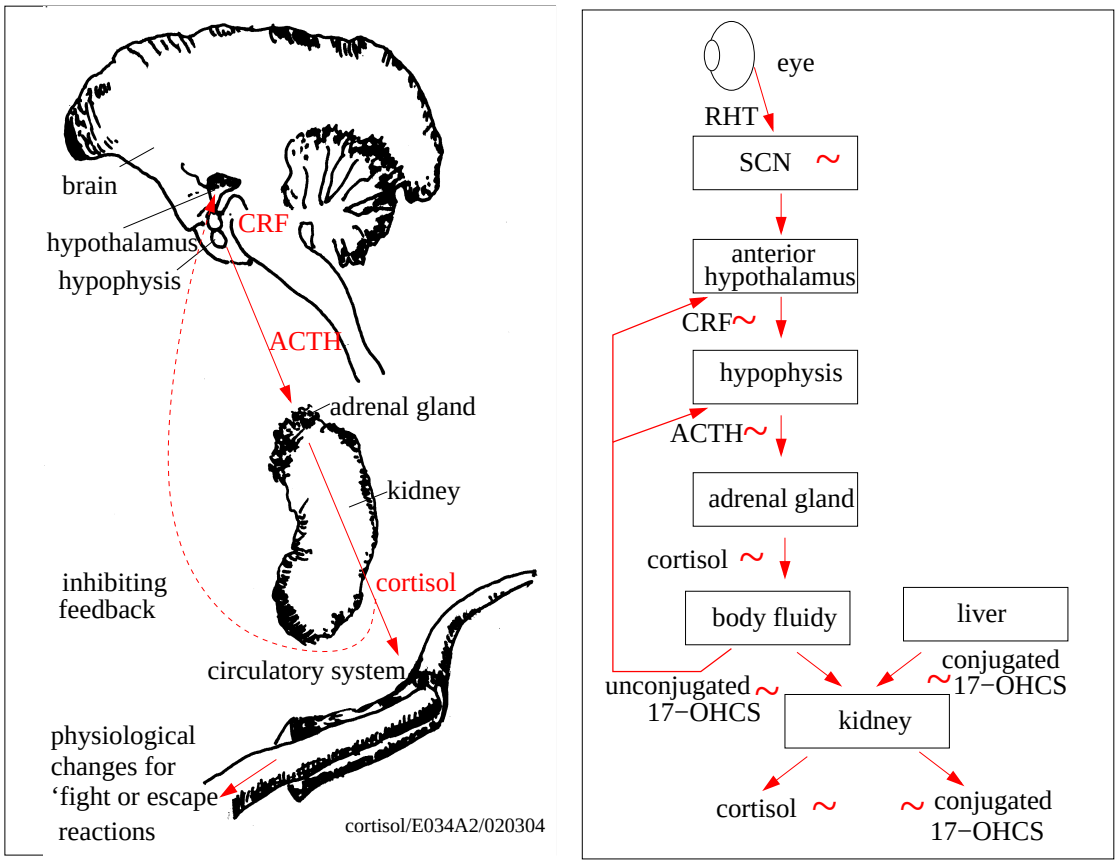

Figure 2.13: *Control of cortisol secretion via the hypothalamus-hypophysis-adrenal-axis. The light-dark-change is received via the retina of the eyes. Signals are conducted to the SCN and synchronize the circadian rhythm of its pacemakers. SCN signals induce the posterior hypothalamus to secret cortisol-releasing factor (CRF) in a daily and ultradian way (and also directly in stress-situations). CRF causes the secretion of the adenocorticotropic hormone ACTH in the anterior lobe of the hypophysis. ACTH triggers cortisol secretion in the adrenal gland. It reaches via the blood circulation different targets (eosinophils, plasma, air ways) and causes in stress-situations 'fight-or-flight'-reactions. By feedback cortisol inhibits the hypothalamus and the hypophysis. Cortisol and corticosteroids are metabolized in the liver and excreted via the kidney. ~: circadian rhythm. After [1029]*

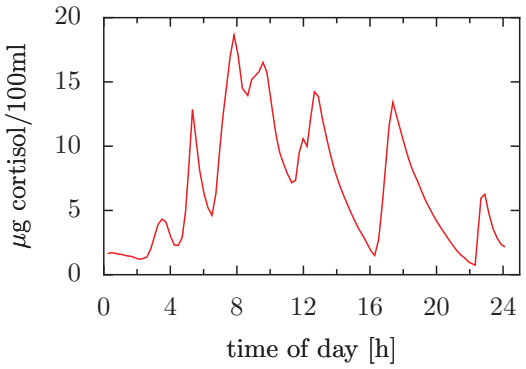

Figure 2.14: *Time course of cortisol concentration in the blood plasma of a person during the course of a day. Every 20 minutes blood samples were taken with a catheter. After [1029]*

hours of the sleep. Its time course is independent of cortisol and insulin. Aldosterone and prolactin fluctuate around 50%, the maxima and minima are found at the same time as the one of cortisol. Testosterone and thyrotropin fluctuate by less than 20%. LH and FSH show especially pronounced ultradian pulses. LH secretion exhibits in woman a menstrual and a circadian rhythm.

Daily rhythms influence the time of birth. There are more babies born between 3 and 4:00 o'clock. If fertilized eggs are implanted in woman, it is successful only between 22 and 24:00 o'clock (in 4 of 79 cases).

2.6 Monthly rhythms in man

The reproduction of man is, as in other mammals, controlled in an infradian way. Estrus cycle and menstrual cycle are typical for mammals (figure 2.15).

Furthermore a circannual cycle in mammals makes sure, that the rutting season occurs at the right time of the year thus insuring that the pubs are born and brought up at favorable seasons. In man the influence of the season on reproduction is low, but established.

Menstruation is a bleeding of the uterus which lasts in woman 3 to 5 days. The uterus mucosa (endometrium) is shed.[8] The ovarial and uterine cycle in woman is 29.5 days as an average. Since the moon cycle is 27 days (sideric) respectively 29.5 days (*'synodic'*, since the earth is moving too) it is tempting to assume a relationship between menstruation cycle and moon cycle. This is, however, not the case. Human menstruation is independent on lunar phases and days of the week ([1167]). However, in monkeys of equatorial South America such relations were indeed found. The menstruation occurs at times of new moon, 14 days later at the time of full moon ovulation and conception is taking place ([402], [403]). Whether this offers a selective advantage or whether it is the result of social effects is unknown.

Although in man no constant phase relationship between moon cycle and menstruation cycle was found, there are some indications that the menstruation cycle among woman can be synchronous (see special topic section 20.9). Other observations speak against it ([1568], [694], [1487]).

Is there also a sexual cycle in human males? It was found that the secretion activity in the auxiliary glands of males occurred parallel to the menstruation cycle of woman ([328]). Estrogens and 17corticosteroids in the male show an 8 to 10 day rhythm ([416]). According to [953] there is a 4 week rhythm of the leukocytes parallel to the androgen-induced nuclear appendixes in the testicles. [659] found a 4

[8] A model of the menstrual cycle of woman has been described by [123].

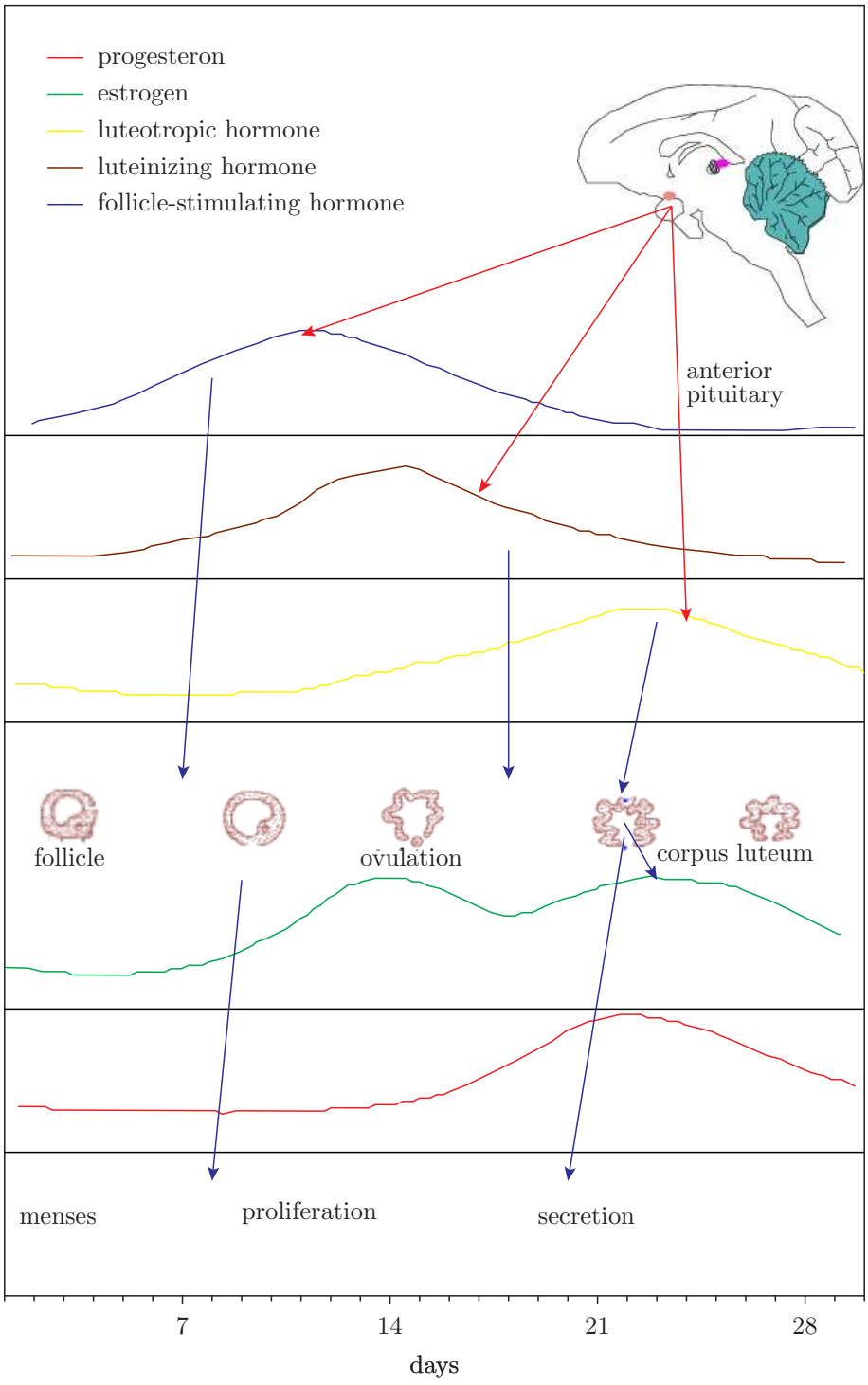

Figure 2.15: *Menstruation in women is controlled by hormones. The anterior lobe of the hypophysis (origin of red arrows) secretes the follicle-stimulating hormone. Later the luteinising hormone is secreted (second diagram) and thereafter the luteotropic hormone (third diagram). How they affect the follicle, is shown in the central part of the figure in sketches. Estrogen is secreted before and after ovulation by the follicle (fourth diagram). Together with progesterone (fifth diagram) it changes the uterus-mucosa (bottom). The stages are shown above the lower time scale (menses, proliferation, secretion). After [1400]*

week rhythm of the urethra cells in the male. The growth of the beard of man fluctuates in a four- weekly rhythm ([771], [770]).

2.7 Organization of the circadian system of man

The circadian system of man and generally of vertebrates is a multioscillatory system. Its structural elements and neural basis are presently studied intensively, mainly in hamsters and mice. The SCN of the anterior hypothalamus and the pineal organ play an important role. In humans the SCN is also a circadian control system, as cases of cancer in this area show: These patients are occassionaly lacking circadian rhythms ([238]). For details of the SCN and its role as a pacemaker of circadian rhythms see chapter 3, section 3.5 and the following sections.

2.8 Models for circadian rhythms

A few models for the description of the sleep-wake-cycle were already presented (subsection 2.3.5). A recent overview on models for circadian clocks is given by [880]. As an introduction see [1574] and [457].

Models can be divided in

1. Mathematical models, e.g. [1555]. They try to describe the circadian system in more general ways (for instance [698], [1507], [879], for a limit cycle approach see [843]) or are designed for specific organisms ([1328], [877] and [878] model the *per-tim* relations in the *Drosophila* circadian system).
2. Structural models, e.g. [1163]: Search for structures, such as a morning- and an evening oscillator.
3. Physiological models (melatonin model of [169], thermoregulatory model of sleep control in humans by [1053]).
4. More recent ones use the new findings of studies of the molecular mechanism of circadian clocks ([504] and [677] for *Cyanobacteria* (see figure 6.19), [27], [269], [1003] for *Neurospora* (see figure 16.16), [289], [664], [865], [1375] for *Drosophila* (see figure 14.15, [7], [485], [699], [1305] for mammals (see figure 3.14)).

The current frontier is represented by models lying between (1) and (2).

In designing oscillator models, single oscillators were proposed first. Later it turned out that they are not sufficient to explain the experimental results. They were therefore replaced by multioscillatory models. Here interactions between the oscillators play an additional role. Examples for such models are

- [1556] used a VanderPol oscillator, in order to describe the circadian system of man. Sleep- and wake times are the result of an equivalence value in comparison with a threshold. If the threshold lies above the equivalence value, the wake period will be short and the sleep period long. If it lies below the equivalence value, the sleep period will be short and the wake period long.
- A model of [397] produces an exact clock by using sloppy components. A discriminator, a threshold and a feedback play a role.

- The Kronauer-model ([831]) consists of two oscillators X and Y, which influence each other to different degrees. X controls the body temperature, Y the sleep-wake-cycle. Six parameters describe the model, of which five are known and the sixth (stiffness of the X-oscillators) is estimated only. In the case of internal desynchronization either the coupling between the oscillators is weak or the period lengths of the two oscillators differ strongly.
  Coupled circadian oscillators were used also in a model by [473] in order to describe phase shifts in jet lag.

- A mathematical model on the basis of the Vander Pol equation with an external force was used to describe jet lag ([536]).

- A feedback model of [717] served to predict jet lag and to compare the predictions with observations.

- A model with a process 1 and 2 was proposed by [1208] (see also [1209]). Process 1 is insensitive toward light. With this model the phase response curve of humans toward light could be described.

- Two oscillator groups with feedback and time delay, in which up to 30 oscillators are coupled ([1508]) simulate the variation of period length, splitting and desynchronization.

- In a further model ([200]) on- and off-cells inhibit each other and stimulate themselves.

- A functional model of different oscillators with a threshold, the variability of which is not too high and not too low, possess a common output. Using a threshold, activity is determined. There is a positive feedback to the coupling device, in which also the Zeitgeber feed ([323]). Using this model, the ontogeny of the circadian rhythm in rat can be described. At a low coupling strength an ultradian, at a high coupling strength a circadian pattern will be found. Light reduces the coupling in animals which are active at night, and strengthen the coupling in dark active animals.

- [1429] proposes a phase model in which the amplitudes are neglected.

- [8] uses a topological model with a limit cycle and a cloud of oscillators.

- Ruoff and Rensing ([1281], [1284], [1283]) use the Goodwin model with three state variables, representing mRNA, the coded protein, and a protein product which represses RNA synthesis. This model can describe besides general circadian properties mutant phenotype, drug and temperature effects.

- [1328] proposed a delay model based on molecular data where the synthesis and degradation of clock proteins and its mRNA are the constituents. This model has been simplified by [879].

- [909] describe the circadian system by using limit cycles and a controlled chaotic attractor. This model explains also the arrhythmic state and the deviations in period length in mutants.

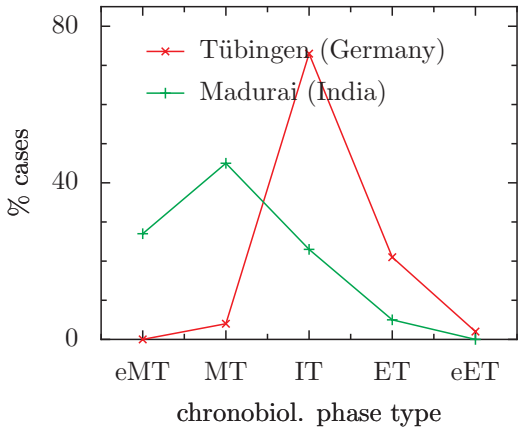

Figure 2.16: *Results of questionaires regarding the chronobiological phase type of humans in groups of students from Tübingen (Germany) and Madurai (South India). IT: Indifference type, MT: morning type, ET: evening type, eMT: extreme morning type, eET: extreme evening type. The curve of the Indian students is shifted towards the morning type*

2.9 Chronobiological phase type

You have surely heard already of morning- and evening types and you probably know to which one you belong. For those of you who do not know, whether they belong to the indifference type (to which most people belong) or more to one of the extreme types, a questionnaire is included in this book which helps you to find out (see section 20.4).

In figure 2.16 the results of this test are presented for a group of students from Tübingen (Germany) and Madurai (India). In both cases a normal distribution is observed. This is expected since the test has been designed this way. However the mean value of the distribution curve lies more to the left (direction morning types) for the Indian students. The reason for this difference is unknown.

2.10 Shift work

Division of labor was certainly found in the prehistoric man already. There were also differences in the daily distribution of activities. Some members of the group stayed perhaps longer awake in the evening than most of the others, whereas others woke up earlier. In this way the group was more protected against attacks during the night as compared to a situation were everybody was sleeping at the same time. Thus there was a selective advantage for a variation of the timing of the group. Already at that early time in the history of man there were besides the main part of the group with a 'normal' sleep-wake cycle 'larks' and 'owls', which woke up earlier or later as most of the group members.

Today security (police, military), social (medicine, transportation, electricity), technological (chemical industry, oil industry, steel industry) and economic reasons (working place and energy are better utilized, which reduces costs) are important reasons for using shift work. I remind you that 20%[9] of the people in industrialized nations do shift work ([1576]), most of them in the industry (partly also in higher positions).

Different kinds of shift work exist: Boom work, 8 or 12 hours shift, rotational- (figure 2.17 and [796]) or permanent shift work ([1291]). The afternoon shift is preferred, next the early shift, and last the night shift. Many people try to avoid shift work and prefer instead a continuous work at a shifted time. Only one third of the shift workers (among them more

[9] 27% of man, 16% of woman ([1028])

woman) do this on a voluntary basis. Most of them are more or less forced into it. In the middle of life the readiness for shift work is highest. For younger people it is easier to stand shift work. With age the willingness to do shift work decreases.

There are a number of arguments against shift work: Health aspects, family ties, social causes, mental stress.

Social, biological and health related consequences of shift work and its influence on circadian rhythms: It is more difficult to adjust to shift work as compared to adjusting to a new time zone after a flight east or west. The reason is, that in shift work the social time situation such as cultural events, television and family life has not shifted. Often the time is too short to adapt to the shift work or it is for other reasons undesired. These reasons are most severe in case of rotating shift work. However, a change between one early, late and night shift (so called 1-1-1 shift schedule) does not change much the pattern of the body temperature rhythm. Its disadvantage is, that during the night shift the body temperature (and parallel to it performance) is low (red curve in figure 2.17). On the other hand it takes a long time until during a three week night shift schedule the body temperature rhythm has adapted to the night shift (not illustrated). Thus, rotational shift work schedules have there advantages and disadvantages and much more studies are needed to find optimal strategies ([1290], [1576]). The individual predispositions have also to be taken into account ([463]).

General adverse consequences of shift work are:

1. The efficiency drops. This has adverse effects on work and sleep. Sleep disturbances occur frequently.

2. The continuous adaptation of the life style to the shift work is a stress for the circulatory system and the digestive system. Heart attacks and digestive tract problems occur more frequently. The circadian system can desynchronize or the phase relationship is unfavorable, changes often or is completely lacking. Meal can not be taken at the normal times. During night work snacks are often eaten.

3. The circadian rhythm is affected adversely: The normal course of the daily routines is work, leisure and sleep. In shift work, however, it is often work, sleep, and leisure. This is likely to be the cause of the difference in the course of body temperature of shift workers as compared to day workers. Furthermore the pattern of shift work is often unfavorable: If a new shift is earlier as the preceding one, it is badly tolerated by the circadian system.

To illustrate some of these points with examples: Russian astronauts have been trained to a 12-hour-day with 6 hours work, 2 hours recovery and 4 hours sleep. On the fourth to fifth day extreme tiredness and vegetative disturbances occurred ([346]). Watches on American nuclear submarines were designed on the basis of a 18 hour day ([1325]). There was a high dropout of the crew members (but not from the officers who did have a normal 24 hour schedule). And a further example: The police in Stockholm (Sweden) worked according to a 20 hour day, which was badly tolerated by the policeman (see in [400]). All these work cycles were a heavy stress, because the cycles used were outside of the range of entrainment of the hu-

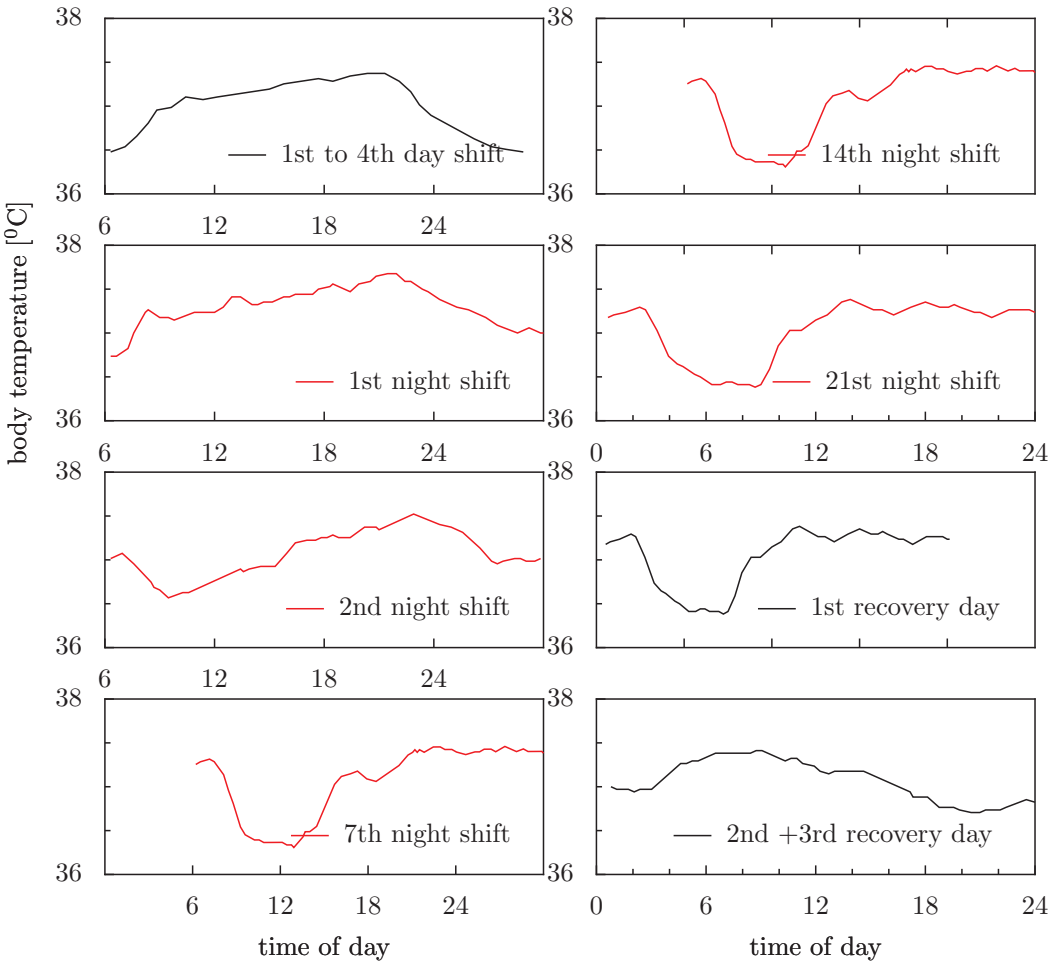

Figure 2.17: *Course of body temperature measured during 1-1-1 shift schedule: After a day of no work (black curve top left, blue horizontal line is sleep time) one day of early shift work from 6:00 to 14:00 (green curve, center left), one day of late shift work from 14:30 to 22:00 (blue curve, bottom left) and one day of night shift from 22:00 to 6:00 (red curve, top right) followed by two days off duty (black curves, right center and bottom). Body temperature values are means from four subjects. After [1291]*

man circadian system[10]. Consequently the people involved were more frequently sick or they even quit work.

Many serious accidents were caused by people which sinned against their biological clock: They were over tired (nuclear power plant accidents in Tschernobyl 1986 and in Three Mile Island 1979, the Challenger-space shuttle disaster in 1986, the accident of the oil tanker Exxon Valdez 1987) or they had fallen asleep (capsizing of the ferry Herald of Free Enterprise at the Belgian coast in 1987, running ashore of the Japanese oil tanker Matsukaze at Seattle 1988). Numerous car- and airplane accidents had similar causes ([1618]).

Rotating shift seems to be especially stressing. It would be better to have a permanent shift instead. This would allow a permanent and fixed synchronization of the daily rhythm. Permanent shifts are, however, so far frowned upon. A number of reasons are responsible for it, among them social discrimination. These disadvantages of shift work could, however, be avoided. Shift workers should get more time off. They should also get enough sleep. It has been reported that engine drivers sleep only 6.5 instead of 8 hours. Although they nap more often, they are still sleep deprived. This can be seen by the fact, that they sleep longer on their days off.

The efficiency is decreased by shift work: The number of accidents increases, the error rate increases (figure 2.18). The efficiency, by the way, parallels the body temperature.

Shift worker suffer under a number of complains: They claim that their social life is perturbed, their health is affected

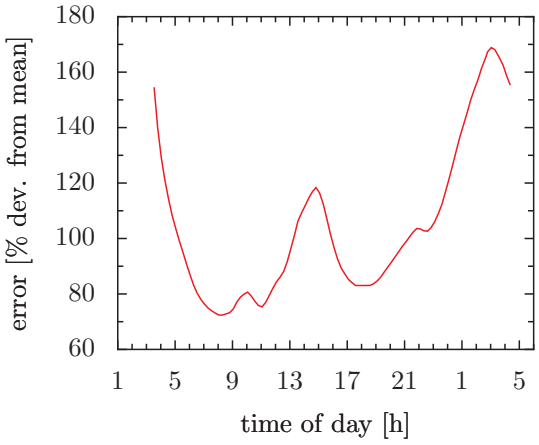

Figure 2.18: *On the y-axis the deviation of error frequency from the mean (in %) is plotted for 62000 shift workers in the industry. After [105]*

and their efficiency is reduced. The rhythmic secretion of digestive enzymes has changed. Sleep is disturbed. One third to two thirds of the shift workers fall occasionally asleep during work. This happens not only during the night shift, but also during the day- and evening shift. Consumption of sleeping pills is high.

Besides the consequences of a disturbed (flat, desynchronized) circadian rhythm there are secondary adverse consequences. They are caused by smoking, coffee and alcohol. Cancer rate is increased, which often does not show up before five years have elapsed. The percentage of sick shift workers is often underestimated because the ones who quit for health reasons are not counted anymore (self selection of shift work). Furthermore shift workers visit the doctor less frequently. Finally the different kinds of shift have different averse consequences.

How can adverse effects of shift work be avoided or eased? To start with, not all humans are to the same extent able to

[10] range of entrainment lies between 22.5 to 26.8 hours, although depending on light intensity

stand shift work. Younger people take it better as older ones do. Morning types have more difficulties with it as compared to evening types (figure 2.19).[11] Furthermore the surrounding of shift work can be improved. The weekend, for instance, should be structured in the same way as the shift work during working days. That is, if one works evening shifts, one should continue to stay up late during the weekend and go to bed late. Social Zeitgeber should be taken into account. Nor should other Zeitgeber be neglected. Sleep itself is important as a Zeitgeber. A so called 'anchor sleep' of a certain length (for instance from 00-04 o´clock, if normal sleep is from 00-08) has shown to have its merits (page 234 in [1013]). The remaining sleep can than be taken at different times and work hours taken in between the two sleep periods (figure 2.20). Other ways of optimizing shift work have been discussed by [4] and [1022], to name a few. Machines and equipment have been and are constantly optimized, but unfortunately man who serves the machine has not been given enough attention[12].

People belonging to risk groups should not be allowed to do shift work. To

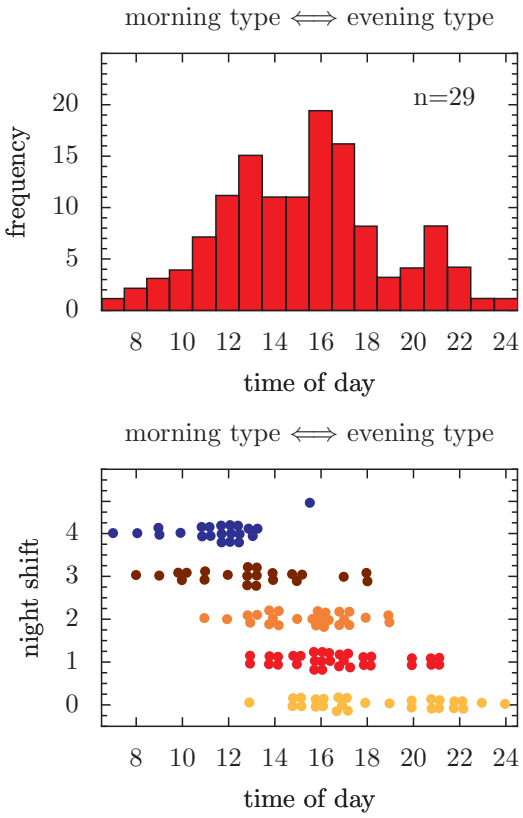

Figure 2.19: *Morning types stand shift work less well as evening types: In 129 nurses of a hospital the chronobiological phase type was checked (top curve). Than these persons were asked, how they tolerated night shift (5 questions). The results (lower part of the figure) show, that morning types tolerate night shift less well as do evening types (4: avoiding night shift, 0: preferring night shift; x-axis: score of phase type, morning type left, evening type right). The correlation coefficient is -0.72. After [636]*

[11] The amplitude of the body temperature of evening types is as a rule larger. They tolerate therefore shift work better, because they adapt less or not to the changed conditions ([1217], [755]). A morning type, however, has normally a circadian rhythm with a low amplitude. In this situation the body tries to adapt to the new conditions ([1116]).

[12] 'No responsible manager would consider operating a piece of machinery outside its design specifications, for that would lead to excessive wear, frequent breakdowns, and early replacement. Yet managers and workers alike have accepted as inevitable the physiological costs of shift work schedules that exceed the design characteristics of the human circadian system.´ ([1027])

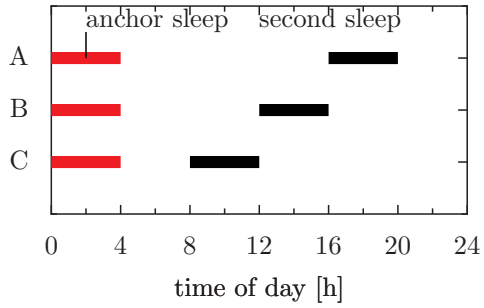

Figure 2.20: *An anchor sleep for a person who usually sleeps from 00 to 08 am would be from 00-04 o´clock. Work hours would follow or precede the anchor sleep, and an additional sleep period could be taken at various times as indicated by the second black bar. In this way disturbances of the circadian rhythm of body temperature are reduced considerably. After [1013]*

this group belong persons suffering under diabetes, respiration- and circulation problems, kidney problems, epileptics, schizophrenics, depressives.

In a review article ([1022]) has put together different ways in which shift workers can be helped (from their employers) or help them self in avoiding these problems or at least reduce them. See also [262]. Certain drugs which enhance alertness can countermeasure to fatigue in shiftwork ([463]).

[365] proposed to organize shift work and night work anew. Shift work should be offered in a way that it would be done voluntarily. Instead of changing shifts a permanent shift work should be introduced and used. The shift worker should be advised in respect to chronohygienic knowledge and the danger of this work should be pointed out to him. There should be uniform shift changes which are coupled to the onset of the school year. This would ease inter-familiar arrangements. If too few or too many workers enroll for a shift, the allowances must be corrected correspondingly. The weekly salary should not change, but there should be more or less working hours. There should be facilities available (for instance with special lighting conditions) in which the circadian rhythm of workers could be adapted to a new shift. The social environment must be made attractive in such a way, that the shift worker sticks to his daily scheme even at the weekend. In late and in night shift bright light exposure during the day should be avoided.

2.11 Jet lag

Everybody who has traveled is familiar with jet lag. It occurs if one passes several time zones by flying east or west and when one has to adapt to the phase shifted day-night conditions (figure 2.21 and table 2.1[13]). In flying north-south the jet lag is lacking. Here only the stress due to flying is encountered.

Jet lag affects the passengers, but also the air crew. For them the jet lag is not only annoying. Their efficiency is reduced and therefore the safety of the passengers endangered. About 65% of all air traffic accidents are due to mistakes of the pilots or the crew ([1029], [1015]).[14]

[13] see also http://www.netcamera.de/wcn/frameset.htm?/info/timezone.htm|contents.htm

[14] Mistakes in manual control are rare. In most cases it is the lack of knowledge, communication errors, wrong decisions, or that available alternatives were not used. The current trend to autocontrol lowers the motivation of the pilots, a further weak point in modern aviation.

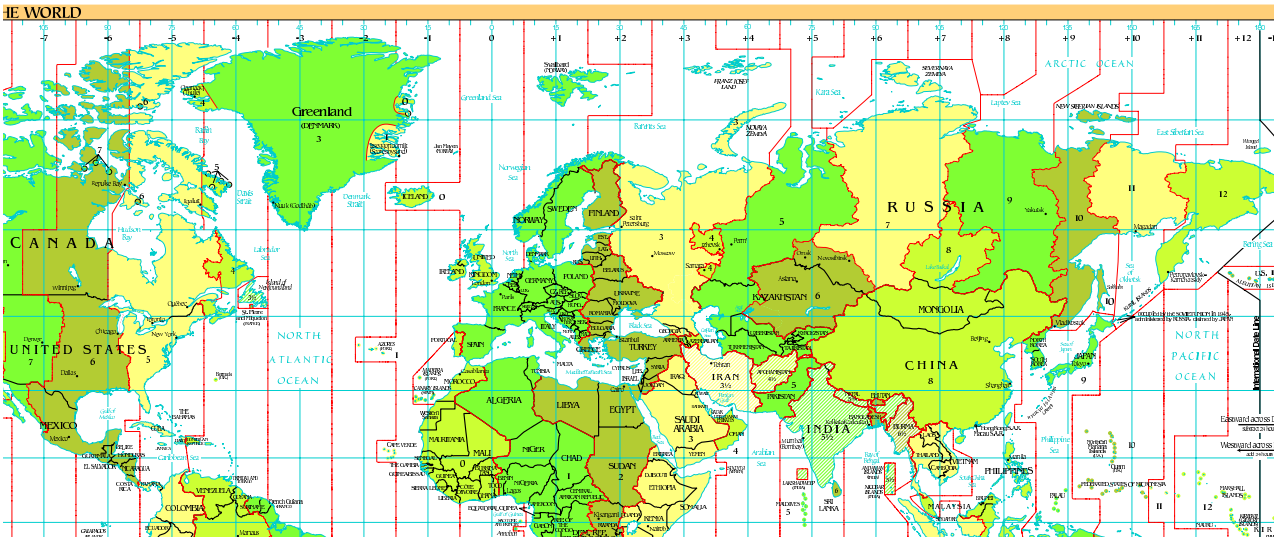

Figure 2.21: *Time zones of the earth. The figure shows the twenty four time zones of the earth (Greenwich, London=0) and the countries belonging to these zones. If it is, for instance 1 o'clock at night in Germany (+1), it is 11 o'clock in New Zealand (+1 to +12 is 11 hours difference). If it is 8 o'clock in the morning in Germany (+1), it is in New York (-5) 2 o'clock in the night (+1 to -5 is -6 hours difference). For calculations see table 2.1). From* The world fact book CIA *under https://www.cia.gov/library/publications/the-world-factbook/reference_maps/time_zones.html*

Table 2.1: *Time zone table. The top row refers to the times given in figure [zeitzonen]. 0 (=24o´clock) would be Greenwich time, 9 Tokyo, 19 Eastern America. If the time at a any place on earth is known, the corresponding time for other time zones can be found by checking the left or right columns (example: If it is 8 o´clock a.m. in Frankfurt, what is the time in New York? Frankfurt has central European time, therefore the column with 1 at the top has to be used. Go to 8 in this column, than in this row to the right until the column which contains the head number 19 (Eastern America). It shows that in New York it is now 2 o´clock a.m.*

22	23	24	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
23	24	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
24	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1
3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2
4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3
5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4
6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5
7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6
8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6	7
9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6	7	8
10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6	7	8	9
11	12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6	7	8	9	10
12	13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6	7	8	9	10	11
13	14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6	7	8	9	10	11	12
14	15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6	7	8	9	10	11	12	13
15	16	17	18	19	20	21	22	23	24	1	2	3	4	5	6	7	8	9	10	11	12	13	14
16	17	18	19	20	21	22	23	24	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
17	18	19	20	21	22	23	24	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
18	19	20	21	22	23	24	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
19	20	21	22	23	24	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
20	21	22	23	24	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
21	22	23	24	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Jet lag is due to stress and to effects on the circadian system as a result of traveling through time zones. Before the circadian system has adapted itself to the phase shifted day it undergoes transients. During this time the amplitude and the phase position of the circadian system is changed or is temporarily internally desynchronized. The causes and symptoms are similar to the one in shift work.

The consequences of a reduced efficiency after a long distance flight crossing time zones is documented by the negotiations of Dulles in 1950 in Egypt. The American foreign minister had crossed 6 time zones to the east and was badly adjusted with his endogenous clock to the Egypt time. As known, the Assuan dam project was taken over by the former Sowjet Union. Diplomats, business man and sportsmen are also strongly affected by such flights crossing time zones.

For the traveler who stays for some time at the destination country the adaptation is easier as compared to a shift worker, since the social Zeitgeber in the country help to adapt quickly (first described by [1373]). One should try to adapt to the new time zone already well in advance of the actual flight by shifting each day the phase of the circadian rhythm. This adjustment occurs faster or slower, depending on the behavior of the passenger. Zeitgeber help to become synchronized at the target of the journey. Strong time cues are the light-dark-cycle ([285]), the timing and kind of meals[15], but other Zeitgeber play also a role such as drinking, medication[16] (see section 20.8), activities such as jogging ([1045]), personal factors such as life style, motivation[17], emotional behavior, professional experience, routine, geographical and ecological factors (climate, altitude), operational factors such as begin and duration of work.

In contrast to the passenger who tries to adjust to the new time zone, for the crew which flies back soon, and for the passenger who has only a short stop, the situation is different. This asks for a different strategy. Both groups should not adapt after a time zone flight[18].

There are recommendations for the air crew personal for their resting time in flights crossing time zones. The ICAO (International Civil Aviation Organization) has come up with a formula for time of rest under such long distance flights. According to it the time of rest per 2.4 hours (=1/10 day) can be calculated (table 2.2). However, individual adaptations are necessary. Unfortunately there is still a lack of detailed information. It would be important to record body rhythms during the flight, to have notes regarding the sleep and tiredness. From these informations conclusions could be drawn how jet lag can be avoided or reduced. So far there are only a few physiological and biochemical data. These studies could also be performed on the ground (for instance [1556],

[15] Phase shifts by combining multiple Zeitgeber. Food with high protein content promotes catecholamine synthesis (wake-time), high carbohydrate content promotes serotonin synthesis (sleep time). Methylxanthine containing drinks such as coffee, tea, cacao are also chronobiologically active substances ([366]).

[16] Jetlag-pills: Melatonin ([22]), benzodiazepin (a sleeping drug) inhibits GABA in the SCN, triazolam ([1492]).

[17] Too much attention is paid to the pilots. Instead the whole crew should be taken into account. Group dynamics, style of leadership, personality structure of the crew, kind of communication are important.

[18] The Aeroflot provided their pilots sleep in complete isolation and tried to avoid night flights, if possible.

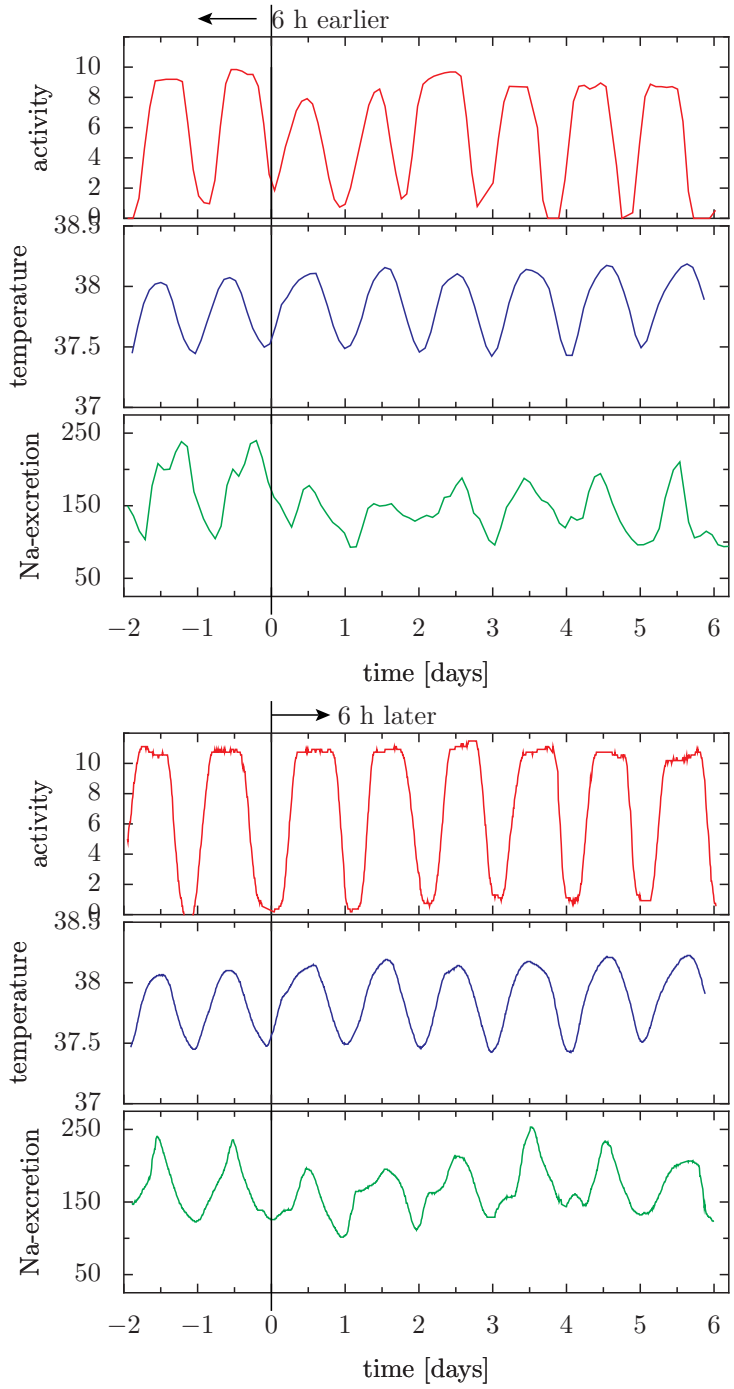

Figure 2.22: *Adaptations of body functions after east flights (top curves for activity (red), body temperature (°C, blue), and sodium excretion in urine (mg/h, green)). Adaptations of body functions after west flights (bottom curves). Flight indicated by vertical black line. After [1556]*

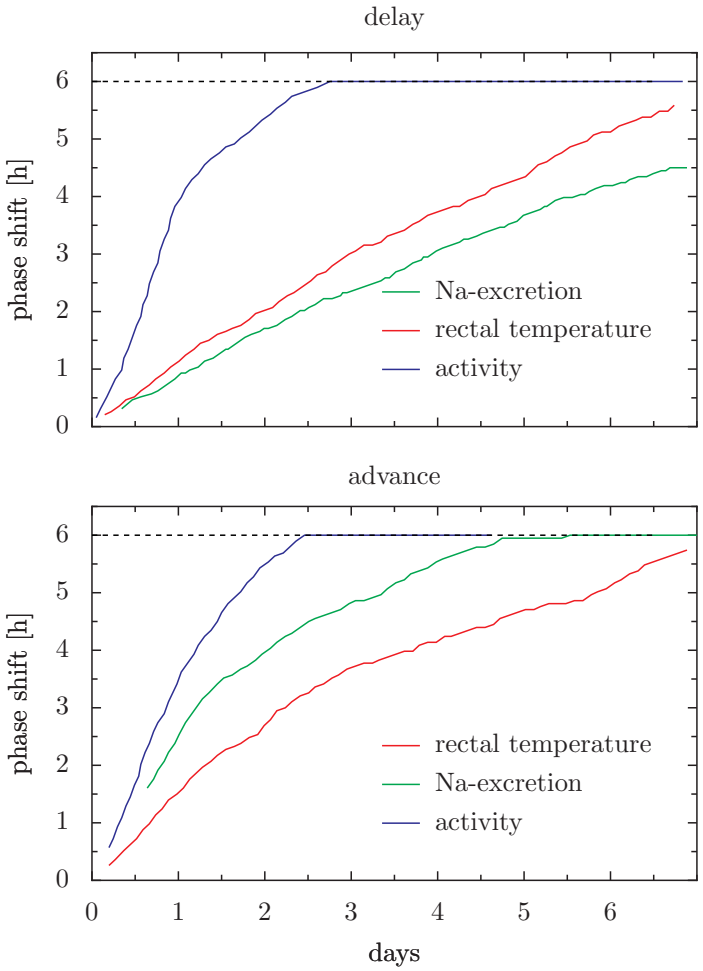

Figure 2.23: *Adaptations of body functions after flights: How long it takes until different body functions (activity (red), rectal temperature (blue), and sodium excretion (green)) adapt to the new time zones after east- (top curves) and west journeys (bottom curves). After [1556]*

Table 2.2: *Recommendations of the ICAO (International Civil Aviation Organization) for the air crew for times of rest. Formula for time of rest under long distance flights according to which the resting time per 2.4 hours (=1/10 day) can be calculated:* $R = Rh/2 + (ZZ - 4) + K_{ab} + K_{an}$ *in which R is the time of resting per 1/10 day, Rh the hours traveling, ZZ the number of time zones passed,* K_{ab} *a coefficient for the time of departure and* K_{an} *a coefficient for the time of arrival. These coefficients can be read from the following table:*

local time	8-12	-18	-22	-1	-8
K_{ab}	0	1	3	4	3
K_{an}	4	2	0	1	3

[1012]). Simulations using models can be helpful ([473], [784], [716], [536]). For instance, there is a difference in adapting to new time zones depending on whether one flies east or west (figure 2.22 and figure 2.23).[19] In a publication it was reported that depressions are more often observed in travelers flying east. Basis of these findings are medical treatments at the airport of Heathrow near London ([695]).

Here some proposals for a safer flight: The air crew should avoid taking sleeping pills and alcohol. It is known that 40% of the stewardesses take medications against sleep disturbances. They affect the course of the sleep negatively. Hangover symptoms occur. Alcohol used in the evening excites, but afterward hypnosis is induced. Sleep is not restful, not deep, the sleep pattern is disturbed, the REM sleep reduced, the general well being negatively affected. These effects can be intensified in connection with other medication. Autogenic training helps to regenerate. Important is also the motivation, the degree of wakefulness, self discipline, the lifestyle and sleep hygiene. Short sleep times (6 hours) are already sufficient for regeneration. The sleep should occur in short flights in isolation, in order to avoid influences of Zeitgeber at the destination country. In this way one continues to stay in his own body time.

2.12 Medical aspects

In the preceding sections we have seen some medical aspects in connection with shift work and jet lag, in which circadian rhythms are disturbed and health is affected. Sleep disturbances are among them. More about medical aspects are presented in this section (see for a recent article [262], [438]).

Since the sensitivity of the body toward many medications changes in a daily pattern, it is not unimportant at what time medicine is taken. This is especially important in the case of medications which

[19] [1556] simulated transcontinental flights in a bunker. The subject did not know about it and was not aware of it. In these experiments the adaptation to westbound flights took longer as compared to east-bound flights. In the east-bound flights the amplitudes were reduced by 53%, whereas the α/ρ-ratio was unaffected. In one case there was a 18 hour delay instead of a 6 h advance. The transients of the shifts are, depending on the observed hand, different: The activity rhythm needs only 2 to 3 days, whereas the body temperature takes 6 and more days. The speed of adjustment depends also on the strength of the oscillator. The efficiency is lower after advance shifts, not so after delay shifts. This agrees with experimental results of flights. Not in accordance with experimental results are the findings in the bunker experiments, that advance transients take less time as compared to delay transients. This is, however, in accordance with Wever's model and his animal experiments. It does not depend on the sequence of the flights and not from the time of day ([785]). Perhaps the strength of the time cues is of significance or the stress of the flight.

exert also a toxic effect as for instance in cancer therapy. Times of the day have to be found at which the substance is less toxic, but most effective against the cancer. Anesthetics, analeptica, corticosteroids, anabolic steroids, histamines and alcohol are further examples for substances, the effect of which changes in a diurnal way. Chronopharmacology has become in the meantime a research area by its own and their results have to be taken into account by medical doctors while describing medication and treatments ([881], [636]). Likewise in surgical treatments these periodic changes of sensitivity play a role. Pain is received differently at various times of the day ([723]). The heaviest pains are felt around 18 o'clock, whereas during the night and in the morning they are weaker. Since sensitivity against pain is centrally controlled, painful operations as for instance in the treatment of teeth should be made in the morning. Unfortunately the efficiency of the dentist is at that time not optimal (it reaches its optimum in the afternoon).

Endogenous depressives[20] have a lower concentration of the monoamines serotonin and noradrenalin in the brain. The density of the noradrenalin-receptors in the cortex is increased in compensation. The hypothalamus-hypophysis-adrenaline axis is deregulated, because by increased stress (genetic disposition, problems during childhood) more CRF is secreted. As a consequence more cortisol ('fight and flight hormone') is formed ([1068]) (figure 2.13). There are a number of indications that the circadian system can be involved in endogenous depression (subsection 2.12.3, [549]). It was assumed that a part of the circadian system runs faster. Therefore this rhythm can not -as the other rhythms- be synchronized to the 24 hour day.

[20] 5-12% of men and 10-20% of women in the US underwent at least onces during their life a major depressive episode, half of these more than onces. In the US 30800 persons per year permit suicide. The costs amounted in 1992 to 43 Billion dollars.

2.12.1 Chronopharmacology

Otto Loewi discovered the chemical transmission of nerve impulses in a dream in 1920. On the next morning he had forgotten the details. In the next night he had the same dream. In order to avoid that he would forget again the details he performed the experiment straight away during night at 3:00 in his laboratory: He stimulated the vagus nerve of a donor heart of a frog and demonstrated that by this treatment the heart rate of a recipient heart was slowed. If he would have done this experiment at an other time the difference would have been much smaller or even insignificant, since this event is modulated by a daily rhythm. Together with Henry Dale he obtained for this discovery the Nobel price in 1936.

The body reacts to substances administered from outside quite differently, depending on the time of day. The pain appeasing effect of novalgin, for instance, changes in a daily rhythm (figure 2.24). The effect of antihistaminic drugs stays for 15-17 hours if the substance is given around 7:00, but only 6-8 hours if given around 19:00 ([1215]). Digitalis has at night twice the effect as during the day. Glucocorticoids are more effective during the day as compared to the night and show less side effects. Aspirin, appetite curbing substances, sedatives (barbiturates), amphetamines, endotoxins, poisons, but also x-ray irradiation have all different effects

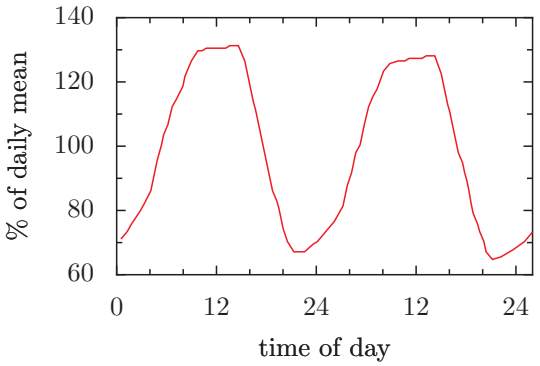

Figure 2.24: *The pain appeasing effect of novalgin (percent of the daily mean) is higher in the morning and early afternoon as compared to the evening and night. After [636]*

at different times of the day. The optimal time for medication against cardiovascular, endocrine and other disturbances depends likewise on the time of application. Medication against too low blood pressure should be taken in the morning, when the blood pressure is at its lowest point. However, medication against too high blood pressure should be taken in the evening, when the blood pressure is highest.

The toxicity of a substance can vary considerably. Neostigmin for instance has during the night a toxicity which is 50% higher as compared to its day effect. A chemotherapeutic against cancer, cytosine-arabinosid, has less toxic side effects if given at different times of the day in varying amounts as compared to the same dosage every three hours as was done before ([588]). The time of application of medications can and should thus be optimized. Undesired side effects of the adrenal hormone and synthetic corticosteroids can be reduced by the proper timing of administration: In this case it would be at the time of waking up. At that time the adrenaline-secretion is maximal. In testing pharmaca the time of day has also to be taken into account. A high variability in studying the effects of pharmaca is partly caused by differences due to the daily rhythm.

On the other hand medications can also influence the rhythm. Quiadon, a sedative, which however increases activity and efficiency, is an example ([1389]). Melatonin affects also the circadian rhythm (see figure 3.17 and [890]). These substances help to adapt more quickly to phase shifts as a result of traveling through time zones. It is also conceivable that with a special chronotherapy only certain rhythms are manipulated, whereas others stay unaffected. If internal desynchronization would cause certain diseases, the normal phase relationship could be re-installed by such pharmaca.

For more details see [1214], [1216], [881].

2.12.2 Seasonal Affective Disorders and light therapy

Besides endogenous depression there exist also depressions which are known as 'Seasonal Affective Disorders' (SAD). Here we are dealing with a disease which is less severe and less noticeable as compared to endogenous depression. Its distribution is heavily underestimated, because the affected people will seldom see the doctor. SAD was described in 1982 by [1266]. The disease begins in the late fall and winter (October to December in the northern hemisphere) and ends in the spring (March). In most cases it is a mild depression. There are, however, also severe cases. This depression depends on the latitude. It is found often in persons who live in higher latitudes in the northern and southern hemisphere ([1462], but see also [947] and references therein). The follow-

ing symptom are characteristic: The activity of the patients is reduced, they go to bed earlier and rise up later; sleep is thus prolonged. The sleep structure has changed: Latency is longer, REM density increased, the delta sleep reduced. These patients have difficulties to concentrate during their daily work. They lose interest in sex, eat more and gain weight. They are shy, withdraw from their physical and social environment. The neuroendocrine system is, however, normal which stays in contrast to the typical depressive person.

During the winter they show 'hunger for light' ([1543], [854]). SAD patients are more sensitive to variations in the length of the natural day ([534]). They are super-sensitive to light during the winter ([1464]). It was proposed that changes in photoperiod induce SAD and that the duration of melatonin secretion mediate the effects of photoperiod on behavior (see [1539] and section 13.6). The effects seem to be more pronounced at higher latitudes (review [853]). A photo-therapy with bright light during the winter shifts the winter behavior to a summer behavior in 60% of the patients. It thus seems to be effective in treating the SAD disease ([1466]). The effect is mediated by the eyes ([1544]). It is assumed that light therapy in SAD-patients acts via melatonin. The melatonin pattern differs between summer and winter in patients, but is the same in unaffected individuals ([1540], see however discussion in [947]).

Different photo-therapies were used. According to one method the patients were illuminated with light of 25000 lux for 3 hours in the morning before sunrise and in the evening before the usual time to go to sleep. The depression improves after 2-4 days. The same effect was gained by 1 hour light with 1000-2000 lux two hours before the normal wake-up time. Light in the evening is also effective. There are, however, also reports of experiments which speak in favor of a placebo effect of the light. There were no significant differences in the effect of weak (30, 400 lux) and stronger white light (6000 lux) and between weak red light and stronger white light, which was administered in the morning to SAD patients ([1264], [700], [1461]). It does not seem to matter whether the light is applied in the morning, at noon or in the evening ([891], [985], [1468]); see however [875]. But the treatment should exceed one week. Preferably it should be applied for three weeks ([349]) and might need to be continued throughout the winter. Dawn simulation improves the efficiency of the light treatment ([983]). The most favorable effect of the light therapy is found at the lower range of high temperatures.

Another form of SAD is known where the patients become depressed in the summer ([1543]). It has been much less studied than winter SAD. Prevalence of summer depression seems to increase with *decreasing* latitude. It was suggested that heat and not photoperiod may be responsible for this type of SAD ([854]). The similarities between seasonal patterns of human reproduction and of seasonal affective disorder suggest a common biological cause of these two forms of seasonality.

It is interesting that the main season of conception for children of SAD patients is in the late summer (studies on 219 patients in the United States), whereas normally the maximum is found in December. SAD was therefore regarded as a remnant of a seasonally dependent reproduction ([1170]). In human societies it might have been advantageous to withdraw at times of food shortage, to have no children during this time and to reduce the energy consumption. All this are symptoms of SAD-patients. Eskimo women do even today not menstruate during the winter.

What is special about SAD? Is it a disturbance of the synchronization of the circadian systems?[21] Or is the circadian

[21] Does for instance the retina of SAD patients show

system changed, for instance desynchronized, changed in amplitude or phased differently (advanced or delayed) in respect to the norm ([178], [816], [1471])? A light therapy ([856], [1141]) or outdoor light ([1579]) would in this case re-initiate or re-synchronize the rhythms. A phase instability in SAD has also been discussed.

Furthermore, in SAD patients the serotonergic system of the brain seems to be distorted (subsensitivity, [1344], [1078]). Administration of serotonin uptake inhibitors is an effective therapy ([1473]). However, both the light and the serotonin uptake inhibitor treatment does not work in severely ill patients ([1343]).

The course of melatonin concentration in the blood differs between winter and summer. In SAD patients the illumination during fall and winter might not be sufficient to suppress melatonin.

SAD seems to be heritable (see references in [947]).

Many questions concerning the relations between light, SAD, and the circadian clock remain unanswered (see [886], [867], [984], [947]). Several types of SAD are found. Some of them react poorly to light treatment ([1465]). For special literature on SAD see [1079], [1578], [1619] and two articles in [1482]. For practical aspects of therapy see [853], [856], [1265], and [286]. Recent reviews are [947] and [1142]. The effect of light is reviewed by [852]. See also the web-site of the Canadian Consensus guidelines for the treatment of SAD ([855]).

special features? Or are social Zeitgeber too weak to synchronize the rhythm in these patients?

2.12.3 Endogenous depression and lithium-salts

Much more severe as compared to SAD are endogenous depressions. The patients feel sad, hopeless, pessimistic, guilty, are often self-preoccupied and avoid social contacts. Energy, activity and libido are reduced, concentration and memory impaired, sleep is disturbed (see page 433).

According to one hypothesis they are caused by disturbances of the coupling of the two oscillators of the circadian system: They are out of phase. The cause of the disturbed phase relationship is according to [829] an oscillator which is too fast (period length only 21.8 hours). This disturbs the sleep pattern, and the maximum of the body temperature lies earlier. The depression occurs if the maximum of the body temperature is after midnight. Mania occur, if the maximum is in the afternoon or in the evening. The sleep duration depends on the phase in which sleep begins. It will be short if sleep begins in the minimum of the body temperature. It will be long, if it begins in the maximum. In healthy people depression like symptoms and anomalous sleep patterns can be induced if they have to sleep at 10:00.

Endogenous depressions can be treated if the sleep begins several hours earlier. The body temperature-rhythm and sleep-wake cycle are then synchronized again with each other. The airport hospital reports from Heathrow ([695]) speak also in favor of this hypothesis.

Endogenous depressions are treated successfully with Li^+-salts. They lengthen in a number of different organisms the circadian clock. It was assumed that they affect the circadian system also during the therapy of endogenous depression in man. We have studied therefore

in an experiment in Spitsbergen (continuous light during the summer), whether Li^+-salts do slow the circadian rhythm of man (see the special topic under section 20.11 and [549]). This was indeed found ([720, 714]). It was speculated, that Li^+-salts affect the coupling between oscillators in the circadian system ([389]). Later it was also shown in monkeys that Li^+-salts slow their circadian rhythm ([1551]).

Manic-depressive patients, especially women, are super-sensitive to light. Depressions are also found more frequently among women. Perhaps women need more light for the synchronization of their rhythms ([760]).

It was proposed in an ethological hypothesis, that the mental disease schizophrenia is perhaps a kind of nocturnalism ([419]). More about it under special topics, subsection 20.10.

3 Clocks of rodents, their hands and how they are set

Rodents offer a number of advantages for studying circadian rhythms. How time cues such as the light-dark cycle synchronize these rhythms, how this light is perceived and signals transmitted to circadian centers is explained. Mutants help to shed light on the mechanisms underlying circadian rhythms. The importance of the SCN as a central clock, its structure and function is described. Outputs of the SCN to other areas of the brain and to the pineal gland with its hormone melatonin will be presented. Examples for circadian rhythms such as locomotor activity, the sleep-wake cycle, disk shedding in the retina of the eye and other events are given. Processes controlled by the circadian clock might feed back to the clock, or the rhythm might be masked by other events. The circadian rhythms can be influenced in a number of ways. The Indian field mouse Mus booduga *is presented as an interesting experimental animal. There are many open questions on the circadian control in mice.*

3.1 Introduction

As other mammals, rodents are also under the control of circadian clocks. There are several advantages to use these animals for studies of their circadian rhythms. Being small, they are easily kept in large numbers in the laboratory or in air conditioned chambers. They reproduce fast. Physiology and behavior are quite well known. Genetic studies can be performed and numerous mutants are available especially in mice.

The locomotor activity of rodents and body temperature fluctuate periodically during the day. Activity can be recorded for extended time spans reliably by using running wheels (figure 3.1, [70]) or wiggle cages. The records are displayed as actograms (see figure 3.19). Records of body temperature can be obtained telemetrically by a probe which has been implanted intraperitoneally. Sleep is measured and characterized by EEG-recordings. Thus, several 'hands' of the circadian clock are available. They can be used to find out the location and functioning of these clocks.

These rhythms are synchronized by the light-dark-cycle. Under constant conditions the rhythm free runs. Light pulses shift the rhythm. Stocks and mutants show different period lengths of their free run. Different treatments and substances might also influence the period.

In rodents the centers have also been studied which are responsible for the circadian rhythms.

Mice, hamsters and rats are preferred subjects for studies of circadian rhythms. All of them are night active animals. We will in the following use the rhythms of mice as examples. First we will follow the pathway which is used by the light-dark-cycle and other Zeitgeber of the environment to synchronize the circadian oscillator. Afterward the localization and

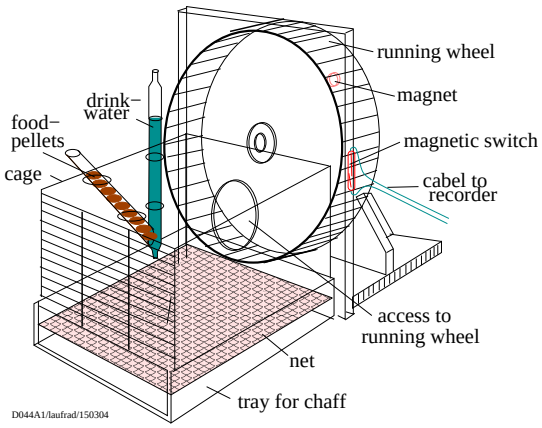

Figure 3.1: *Hamster cage with running wheel for recording the locomotor activity. Water supply from glass tube with nipple, food supply from glass tube with pellets. Wire cage with net on top of bowl with chaff. A magnet is fixed to the axis excentrically which closes a magnetic switch when the running wheel is turned by the animal. Each switching event is recorded and the number per time span (for example every 10 minutes) stored on computer. The data are displayed as a histogram in form of an actogram (example in figure 3.19). After [387]*

physiology of the circadian clock in the suprachiasmatic nucleus of the anterior hypothalamus of the brain is explained. Several mutants are known in which the circadian clock is altered. Their study helps to understand the molecular structure and the mechanism of the clock. How the SCN controls different events is shown in a further section and examples will be given.

3.2 Synchronizing the oscillator by Zeitgeber

The circadian clock of mammals is synchronized to the 24-hour-structure of the environment mainly by the light-dark-cycle. Light is perceived via the retina of the eyes. Whereas in non-mammalian vertebrates extraretinal photoreceptors besides the eyes are used for synchronizing and phase shifting the central circadian clock, in mammals the eyes are apparently the only photoreceptors entraining the clock to the light-dark cycle of the day. Rods and cones are involved, but additionally another component in the retina: A population of retinal ganglionic cells, bipolar cells, called Landolts club, extending through the outer nuclear layer and terminating in the space between pigmented epithelium and inner and outer segments of the photoreceptors ([914], [1499]). They seem to be responsible for sensing the light regime of the environment and its time structure (figure 3.2).

They are evenly spread over the entire retina and project to the SCN, but not to the visual centers of the brain (references in [1183]). These photoreceptor cells are connected with each other either in an adding or in an averaging processor ([449]). The sensitivity of retinally degen-

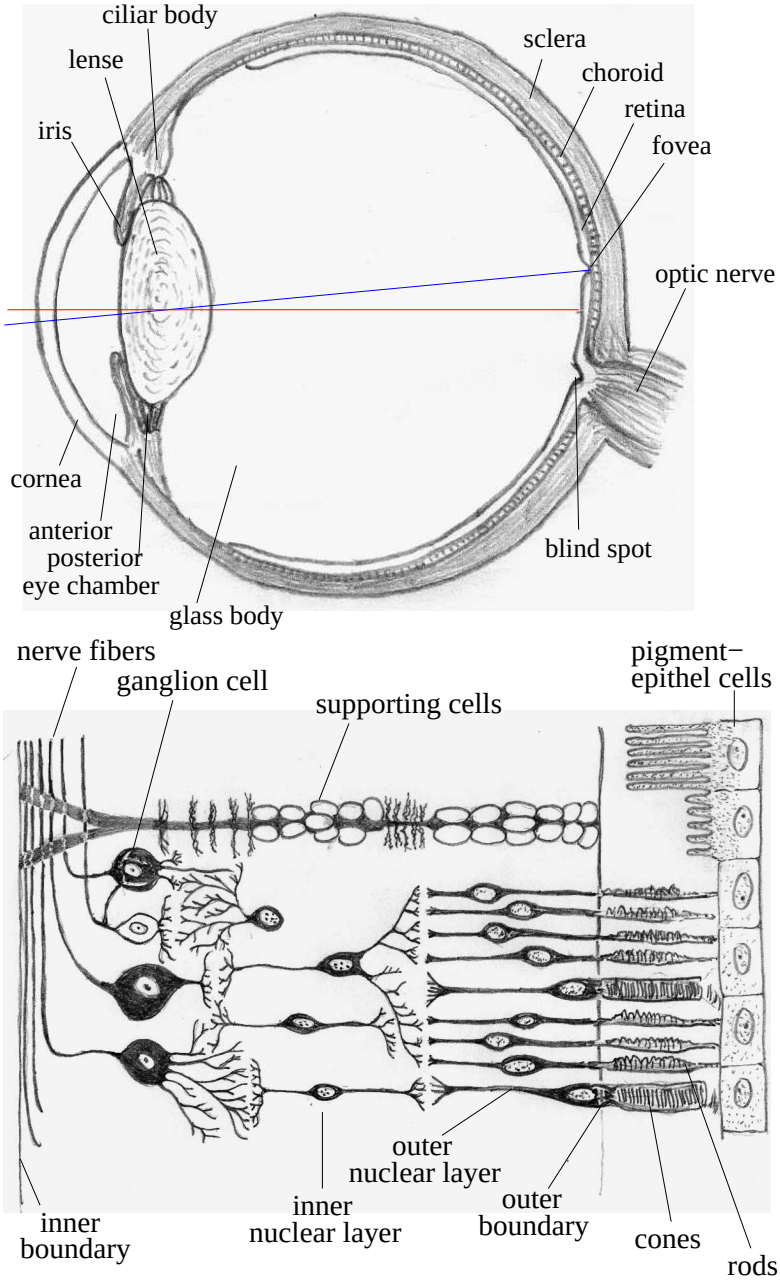

Figure 3.2: *Longitudinal section through human eye (top) with cornea, lens, glass body and and retina. Close-up of retina (bottom) with (from left to right) ganglion cells, bipolar cells, cones (thick and short), rods (thin and long), and pigment cells. After [1034])*

erated mice (rd) for phase shifting light is the same as that of the wild type (which is in contrast to [351], see [450])

In favour of this interpretation speaks also, that blind moles (*Spalax ehrenbergi*) are able to synchronize their circadian rhythm to the light-dark-cycle, although their remnants of eyes are unable to perceive images because the corresponding centers in the brain are absent or extremely reduced ([507]),. The SCN is, however, well developed and contains projections of the rudiments of the retina (reference: [248]).

These ganglia cells might possess an until now in mammals unknown retinal photopigment such as melanopsin, which has been discovered in fish ([1402]) and amphibians ([1183]). The pigments of the photoreceptors which absorb the light which serves to synchronize the circadian behaviour of mammals with the light-dark-cycle, are opsins with cis retinaldehyde as the chromophore[1].

3.3 Pathway of the light signals from the retina to the central circadian clock, the SCN

To synchronize the circadian clock of mammals, light is perceived by retinal bipolar ganglionic cells. They project via a special pathway, the retinohypothalamic tract (RHT), to the SCN (figure 3.3 and figure 3.16). The two other nerve bundles from the eye to the brain, the optic nerve and the accessory optic system, do not influence synchronization[2]. The retinohypothalamic tract was discovered by using autoradiographic methods ([1026]).

Neuronal signals (glutamate as neurotransmitter) reach the SCN and synchronize the pacemaker (see figure 3.14).

The intergeniculate leaflet (IGL) receives also retinal inputs from the retinohypothalamic tract and sends afferent informations through the geniculohypothalamic tract (GHT) to the SCN. IGL-lesions in mice lengthen the period of the running wheel rhythm in continuous darkness, whereas period is not affected in continuous light. The IGL seems therefore to have an endogenous influence on the circadian oscillator in the SCN ([1155]).

In synchronizing the circadian rhythm in the SCN by light signals, transcription factors are involved (see figure 3.4). The exact role of c-Fos is however not completely understood ([1347]).

NO participates also in the circadian control ([1527]): Cells in the SCN of mice show immunoreactivity to NO. NO seems to play a role in the transfer of light-induced signals in the SCN. Furthermore serotonin is involved in the effect of light on the circadian oscillator in the SCN ([141], [1181], [360]).

Not only light, but also the activity of the animals synchronizes the circadian rhythm. If the animals have access to a running wheel for two hours per day only, theperiod length of the rhythm is under otherwise constant conditions exactly 24 hours. That is, the rhythm is synchronized. Locomotor activity increases the serotonin content in the SCN. Serotonin-agonists shift the phase of the circadian clock in the same way as locomotor activity. This indicates that serotonergic afferents are part of the activity-dependent synchronization mechanism ([358], [359], [356]).

[1] 11-cis isomer of vitamin A aldehyde, its all trans isomerization is the first step of phototransduction in all animal visual systems

[2] The circadian system is additionally controlled by other parts of the brain. For instance, if the olfactory bulbs are removed, the period of the activity rhythm is prolonged by 43 minutes. The activity begins 108 minutes later ([1173])

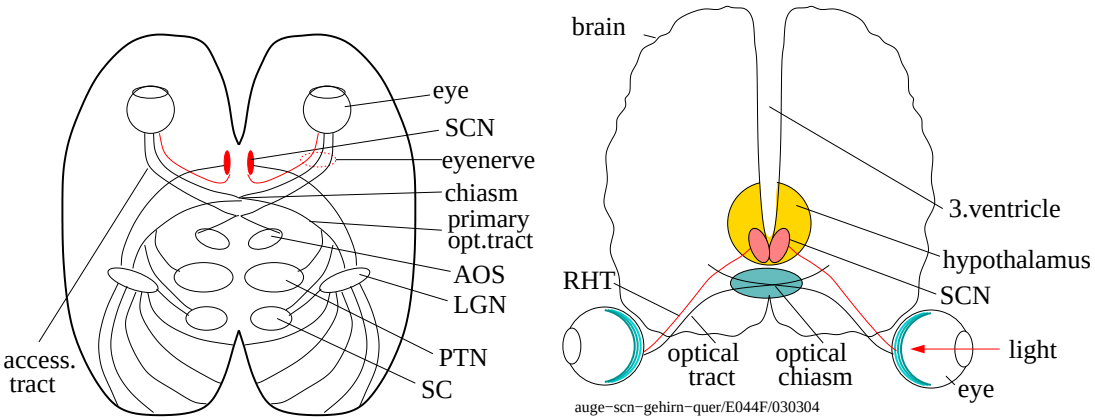

Figure 3.3: *Visual pathways from eyes to brain in hamsters. Left: brain from top. Right: Cross section through brain at the location of the hypothalamus and the SCN. Light is received via the retina of the eyes. Signals are transferred via the primary optic tract, the accessory optic system (not shown) and the retinohypothalamic tract (RHT) to the visual cortex in the brain. The RHT terminates in the suprachiasmatic nucleus (SCN), which contains the 'pacemaker' cells for circadian rhythmicity. After [1029]*

The circadian rhythms are also synchronized if food is restricted to certain times of the day or if the animals are stressed with electrical stimuli ([1014]).

3.4 Influencing the circadian system, age effects

The circadian system of mice can be influenced by, for instance, estrogen application. It shortens the period length in continuous light. If the growth hormone titer is high (in a transgenic mouse), the period is also shortened under continuous light ([426]). Serotonin seems to affect period length of the circadian clock too ([1172]).

Furthermore the circadian system of the animals changes with age. It matures and stabilizes with increasing age ([1545]. In darkness the period of the running wheel activity lengthens with age in mice ([1174], [972]), whereas it is shortened in other rodents ([1174]. Furthermore, with age the circadian system becomes less sensitive to synchronizing light ([83]).

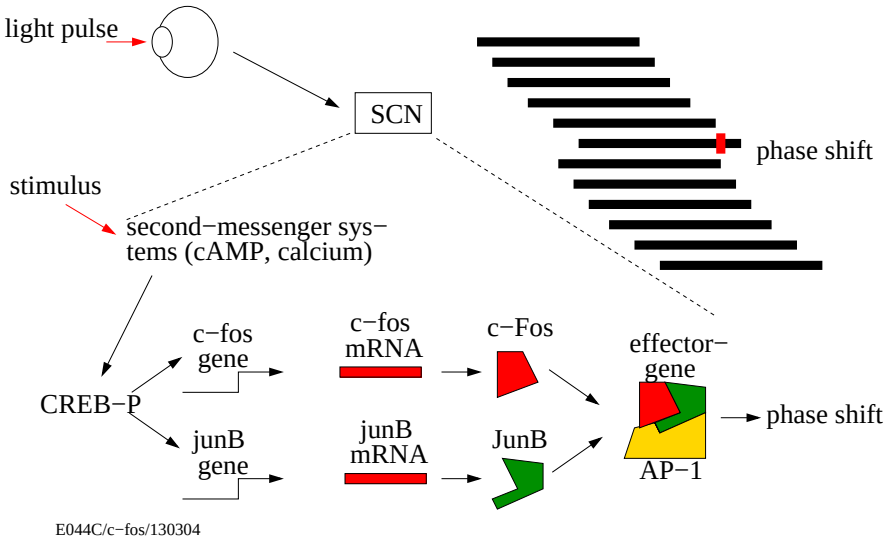

Figure 3.4: *Top: Light pulse is perceived in the retina of the eye, a signal reaches the SCN, phase shifts its circadian oscillators and as a consequence the locomotor activity rhythm (black horizontal bar for each day, red mark: position of light pulse, which advances the rhythm). Below: Signal transduction cascade for synchronization of hamsters by light-dark-cycles. An external stimulus such as neurotransmitter or hormones activate second messenger systems (cAMP, Ca^{2+}) which lead to phosphorylation of cAMP response element binding protein CREB. This is a prerequisite of the activation of immediate early genes IEGs such as c-fos and junB. Transcription (→mRNA) and translation (→c-Fos, JunB) lead to proteins of the Fos or Jun family, which combine to hetero-dimers and combine with the AP-1 region of other gene sectors, activating or inhibiting their transcription. After [1580]*

3.5 Circadian centers

The circadian system of mammals consists of multioscillators, which are arranged in a hierarchic and a non-hierarchic manner.[3] A center of circadian control of vertebrates lies in the paired SCN of the anterior part of the anterior hypothalamus (figure 3.5). For the history of the discovery and newer results see [1533]. Richter has destroyed systematically different brain regions and brain glands in rats ([1232], [1233]) and found arrhythmia, if parts of the hypothalamus were damaged. Before his studies it was already known that the estrus cycle was affected after SCN destruction. These results were re-discovered by [1025] and at the same time independently by [1418].

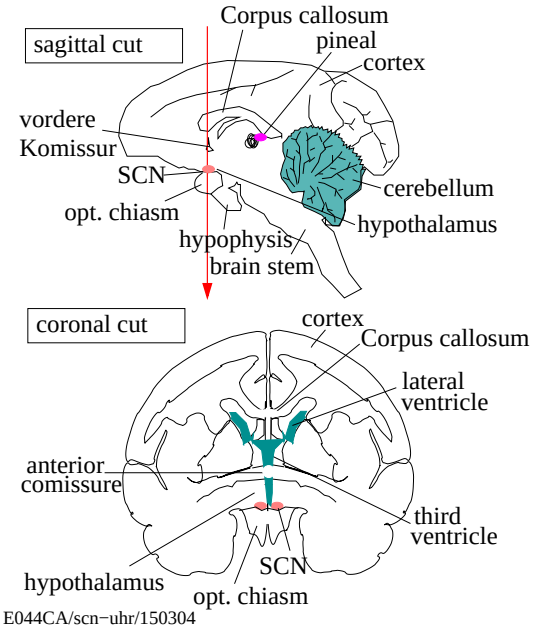

Figure 3.5: *Anatomy of the squirrel monkey brain in mid-line sagittal (top) and coronal sections (bottom; the red arrow shows the cutting plane). The suprachiasmatic nuclei (SCN) are situated lateral to the anterior tip of the third ventricle above the optic chiasm. After [1029]*

In mammals the circadian system is governed by the SCN. It serves as a master oscillator and controls a large number of physiological events and rhythms in behavior. Among them are locomotor activity, sleep-wake-cycle, thermoregulation, torpor, hibernation, functions of the circulatory system and many endocrine events. The synthesis and secretion of

[3] The following findings indicate that a multioscillator system exists ([628]):

- After phase shifts (for instance after flying over time zones) the various circadian rhythms are resynchronized at different speeds. The circadian activity rhythm, feeding, drinking and body temperature rhythms are faster synchronized as are urine parameters ('transient desynchronization')
- In humans spontaneous internal desynchronization is observed under constant conditions.
- In forced internal desynchronisation the range of entrainment is different for the different processes which are controlled in a circadian way. It is likely that in this case multiple subordinated rhythms are decoupled from master centres for some time.
- Internal dissociation ('splitting') under continuous light points to two mutually coupled oscillators. They are in antiphase, that is, shifted by 180^0 against each other. This was observed in hamsters, rats, squirrels, tree squirrels, primates, but also in starlings and lizzards. It is still unknown whether this is due to different oscillators in the SCN or whether it is brought about by oscillators outside the SCN. Perhaps the SCN is specialized for internal interactions of the neurons and their communication.
- Circadian rhythms were observed in isolated tissue, organs and glands (adrenal gland, liver, heart, erythrocytes, intestines ([1029]).
- Cortisol synchronizes urine parameters, but not the feeding and drinking rhythm.
- If discrete neural tissues are distroyed, certain circadian rhythms disappear, wheras others stay intact ([465]).

pineal melatonin is also controlled by the SCN.

If the SCNs are destroyed, the circadian control of all these events disappears. The SCN is not just a tissue, which transfers informations of the light-dark-cycle from the eye to an oscillator somewhere else. In this case its destruction would have prevented *synchronization* of the various circadian rhythms, but the rhythms should not have disappeared. Free run should have occurred instead. But the animals became arrhythmic, if both SCNs were destroyed or lesioned ([1025], [1418], figure 3.9). The circadian rhythm is not affected if only one SCN or a part of it is damaged ([1350]).

Another important indication of the role of the SCN as a master oscillator were recordings of a rhythmic electrical activity in the SCN. This rhythm existed also in the SCN, if isolated from the surrounding tissue by lesions. In the surrounding tissue, however, the rhythm was not found anymore (figure 3.6 and [676]). This proofed that the SCN is an autonomous rhythm-generator which conducts the rhythm to other structures via nervous connections. Recent studies have shown, that substances play also a role which diffuse out of the SCN and control behavior rhythmically ([1381]).

The metabolism in the SCN has also a circadian time course ([1346]). It is high during the day and low during the night (figure 3.7). This rhythm continues in vitro ([1084]), as shown by using 500 μm thick sections through the hypothalamus ([524], [531]). The firing of single neurons can be recorded in the right medium under appropriate temperatures up to three days under constant conditions ([1182], figure 3.8). cAMP can shift the phase of the electrical activity rhythm. Fetal SCNs show already the circadian rhythm in metabolic activity ([1229]). They are synchronized by the rhythm of the mother via different signals, among them dopamin and melatonin (Review: [1228]).

Syrian hamsters, which became arrhythmic by removing the SCN, show a circadian rhythm of behavior again if fetal SCN tissue is implanted ([874] and figure 3.9 for rats). This tissue can also be taken from other species (Syrian hamsters, mice or rats). The induced period length is that of the donor (Syrian hamster, mice). Fetal SCN tissue of rats, however, induces a rhythm with a period which is shorter than that of the donor and recipient ([1397]).

Even cultures of SCN-cells are able to induce weeks later a circadian rhythm in Syrian hamsters, in which both SCN were destroyed. They were implanted in locations of the brain at which the SCN is normally found ([1380]. Thus the structure of the SCN must not necessarily be preserved. If the SCN-cells of two genotypes with different periods are implanted simultaneously in the same animal, the resulting rhythm is coherent. This shows that the cells are able to communicate with each other and to come to an agreement on an average period length. Transgenic cells with markers have been used to characterize the responsible cells ([1197]).

Interesting results are from studies of [1550]. Individual SCN neurons, which were dissociated from each other, were monitored with multi-micro-electrode plates for the electrical activity for extended periods of time. Cultures contained cells with different phases and periods, although functional synapses were present (figure 3.10).

What are the pacemaker in the SCN? Are there different functional parts in the SCN? The SCN of mammals consists of

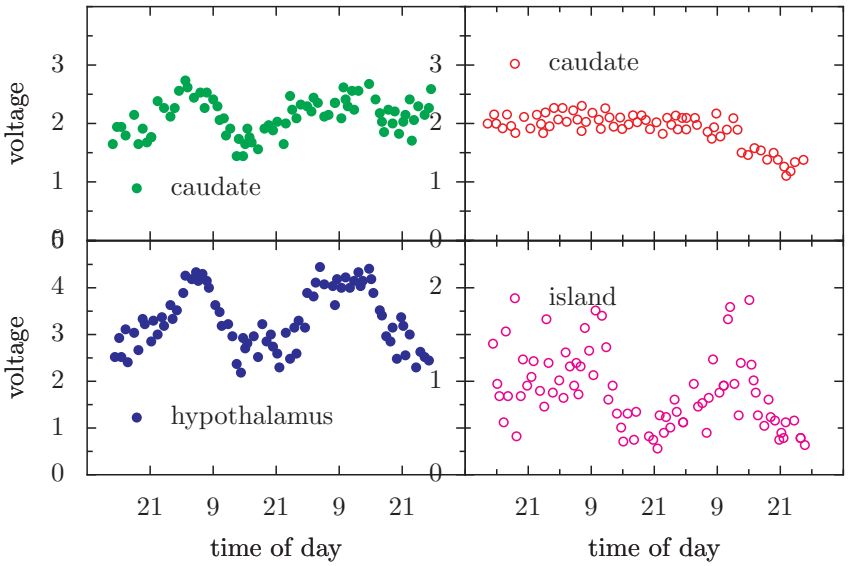

Figure 3.6: *Isolation of the SCN from the neighboring tissue by a circular cut prevents the circadian rhythm of electrical activity outside the SCN (compare top left before isolation with top right, after isolation). The daily rhythm of electrical activity inside the SCN, however, continues (compare left and right bottom; in both cases a circadian rhythm is still present). After [676]*

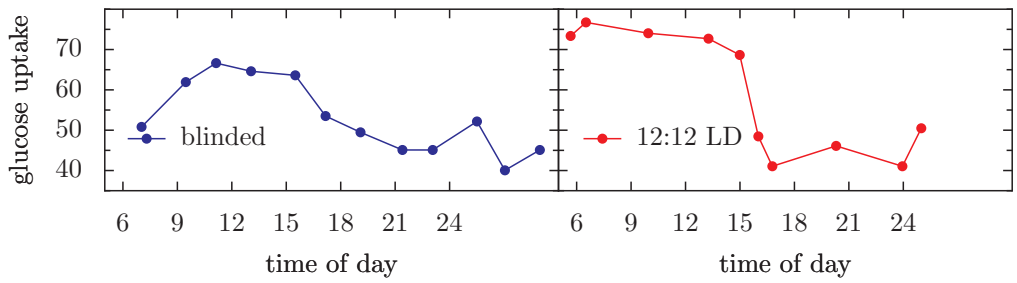

Figure 3.7: *The metabolism in the SCN has a circadian time course, as shown by the accumulation of the glucose analogue 2-deoxi-D-glucose (which is not metabolized, in contrast to glucose) in the SCN during the day phase and low concentrations during the dark phase. Each point represents the results of one animal sacrificed at the indicated times of day. Right curve for sighted animals kept in LD cycles, left curve for blinded animals (that is, in physiological DD). In both cases differences in glucose uptake are found between day and night phase. After [1348]*

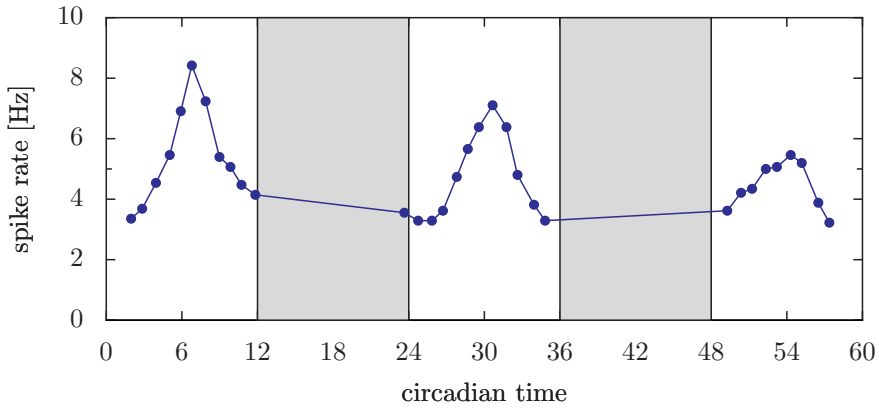

Figure 3.8: *Electric activity in SCN neurons (firing rate (Hz)) in vitro during three cycles (circadian time, x-axis). Mean values of 4 (first cycle), 8 (second cycle) and 3 measurements. Grey area: subjective night. After [1182]*

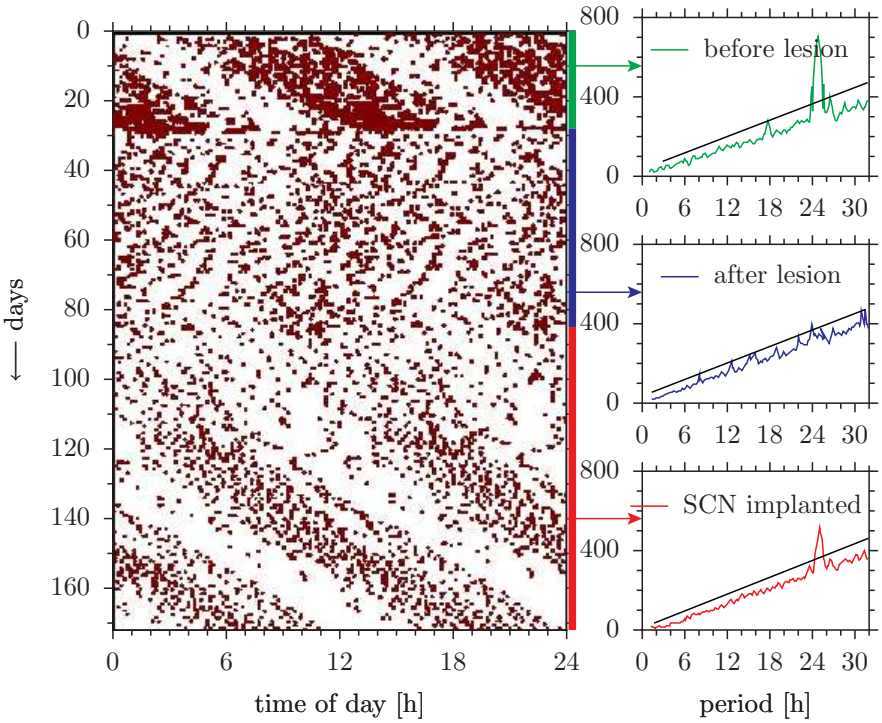

Figure 3.9: *A rat was SCN lesioned on the 28th day of free running in a wheel. It became arrhythmic (marked blue), as shown in the actogram (left). If fetal SCN-tissue is implanted on day 85 (the following period marked red), a circadian rhythm of locomotor activity is exhibited again. At the right, power spectra are shown for the pre-lesion (top, green), post-lesion (center, blue) and post-transplantation periods (bottom, red). The 25 hour rhythm which is significant before lesion (green peak above the significance line) disappears (blue curve) and reappears again after neonatal SCN was implanted (red curve). After [1580]*

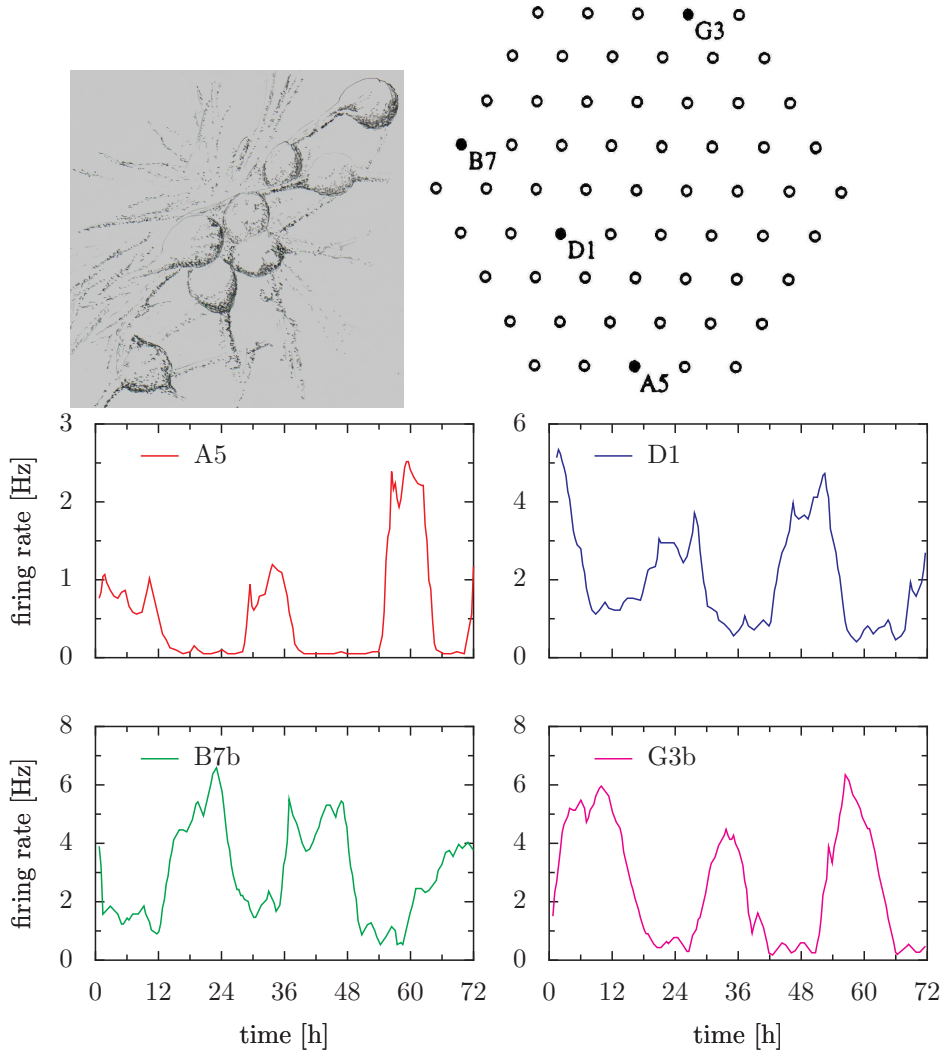

Figure 3.10: *Firing rate of individual SCN neurons, which were dissociated from each other (top left) and four of them recorded for 72 hours on multi-micro-electrode plate (top right, A5, D1, B7b, G3b). Electrical activity (firing rate, y-axis) of these four cells A5 (red curve), D1 (blue curve), B7b (green curve) and G3b (magenda) shown in lower part of figure. Note that cells exhibit different phases and periods, although functional synapses were present. After [1550]*

about eight thousand to ten thousand neurons. If viewed in a *vertical* section, they form a core and a shell whith characteristic neurotransmitters of their neurons, with differing innervation (reviewed in [1024], [410], figure 3.11) and probably with varying functions: The oscillators in the core cells seem to respond to light-caused signals of the retina, whereas the oscillators of the shell cells do not. The core and shell oscillators are, however, coupled with each other and gain in this way a common phase relationship[4]. The electrophysiological activity of *horizontally* cut slices of hamster SCN reveal two distinct oscillating components ([692]). They might reflect the activity of *morning* and *evening oscillators* which has been inferred already earlier from behavioral studies ([1163], [671]). Photoperiodic reactions are supposed to use such evening and morning oscillators, and a long and a short photoperiod do indeed affect the morning and evening peaks of the electrical recordings differently ([692]).

Retinal information is transmitted through the retinohypothalamic tract (RHT, figure 3.16) and via the intergeniculate leaflet (IGL) through the geniculohypothalamic tract (GHT) to the ventrolateral part (*shell*) of the SCN. This part of the SCN contains pacemaker neurons, which fire in a circadian way. The rhythms are light-dependent. In contrast, the dorsomedial part (*core*) receives inputs from non-visual sources and the neurons show rhythms which are not set by the light signals (references given in [665]). The firing rate of the neurons shows a circadian rhythm. High firing rates during the subjective day seem to correlate with peptides and the neurotransmitter gamma-aminobutyric acid (GABA) and standard synaptic interactions are used. However, rhythmic information might also be conveyed by a diffusible substance.

After the destruction of the SCN other rhythms are still maintained, such as the anticipatory food uptake behavior: If mice are trained to food given at certain times, they expect it already before actual feeding. Mice, the SCNs of which were removed, do still show this anticipatory behavior. They must therefore be controlled by another pacemaker center ([954], [137]). Whether the circadian rhythm of the REM sleep is also controlled by the SCN is debated ([1417], [1041], [1588]). It was mentioned already, that a circadian oscillator resides in the retina. There might also be an enteric oscillator. Whether all these oscillators possess the same clock mechanism is not yet clarified. For a review see [1268].

The control of the body temperature is exerted mainly from the preoptic area (POG) and from the anterior hypothalamus (POAH) ([93], [658]). The POAH is a temperature-sensitive area and represents an integration center ([1293]). Lesions in this area disturb the temperature-regulation (see figure 3.12 and [512]). The temperature of the environment influences also the control of the body temperature. Finally, behavior affects the temperature-regulation. Bilateral POAH destruction in rats shifts the average value of the temperature from 37.0 to 38.6^0C. The circadian rhythm of body temperature is, however, maintained, the amplitude of the rhythm even increased by a factor of three. This indicates, that the lesion has influ-

[4] The 'dead zone' of a phase response curve of a rodent to light pulses could be explained by the number of light unresponsive elements which overweight the number of the light responsive ones ([1375]).

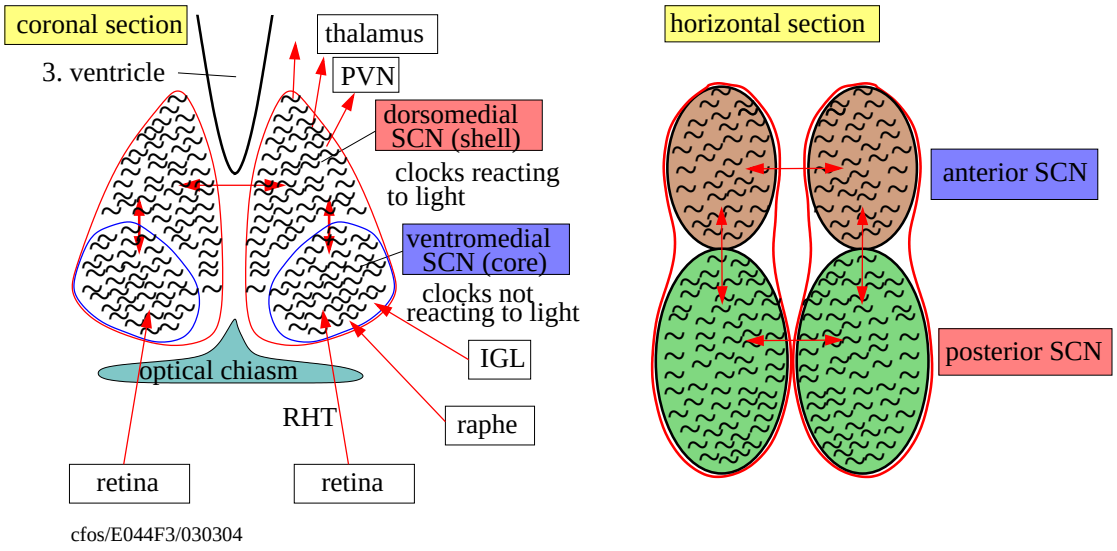

Figure 3.11: *Left: The suprachiasmatic nuclei (SCN) of mammals are paired structures located at the lower tip of the third ventricle and above ('supra') the optic chiasm in the hypothalamus. A coronal section through the SCN reveals a dorsomedial part ('shell') and a ventrolateral part ('core'). Inputs are from the retina via the retinohypothalamic tract (RHT), the raphe nucleus and the intergeniculate leaf (IGL). Outputs run to the thalamus, para-ventricular nucleus (PVN) and other areas of the brain. The shell is supposed to consist of numerous cellular oscillators which do not respond to light inputs. The core is supposed to consist of cellular oscillators which do respond to light signals. Right: A horizontal section shows the anterior SCN (brown), consisting of a population of cells which might represent morning oscillators, and the posterior SCN (green) which might represent evening oscillators. Coupling between the diverse groups is indicated by double arrows. After [1375], [675] and [340]*

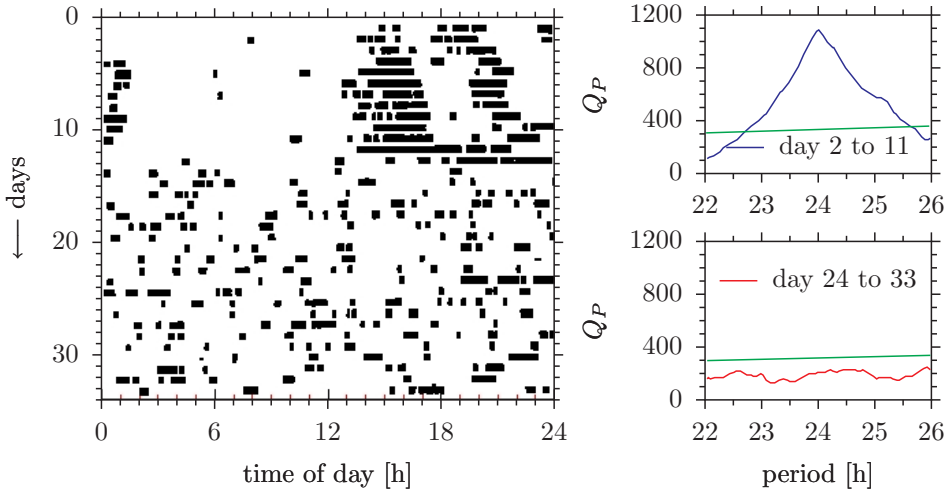

Figure 3.12: *The circadian rhythm of* body temperature *of a Syrian hamster disappears after lesioning the suprachiasmatic nuclei (on day 11). Periodograms for day 2 to 11 at the top right, for day 24 to 33 below. After [1210]*

enced the temperature-regulation, but not the circadian rhythm of the temperature regulation.

The circadian control of temperature-regulation occurs in the SCN and a number of reliable proofs are available. For instance, the circadian rhythm of body temperature (and activity) disappears, if the SCNs are destroyed ([1025], [1418], figure 3.12 and figure 3.13). The mean temperature is, however, maintained and the homeostatic regulation of the body temperature is still functioning.

Selected strains, which differ in various circadian parameters related to the construction of the nest for the litters, possess various amounts of AVP-immunoreactive neurons in the SCN ([177]). In the light-dark-cycle the number of vasopressin-containing cells and their volume fluctuates in a daily and annual period ([649]).

How the oscillators in the SCN control locomotor activity and other events in a circadian fashion, is poorly understood so far (see section 3.5). Figure 3.14 shows how a target cell might acquire circadian control from the SCN both via cytoplasmic and nuclear responses via neuronal signals. Neuropeptide Y as well as serotonin are supposed to be involved in transmitting the signals through afferent neurons of the SCN ([954]). If neuropeptide Y is supplied systemically, the circadian rhythm of locomotor activity of mice is affected ([839]). The figure illustrates also the inputs of light signals from retinular ganglion cells in the eyes via the retinohypothalamic tract. For details see the legend.

What are the target organs of the efferent signals of the SCN? From the efferences of the SCN only the projections to the pineal organ are completely known so far. How other effector tissue receives information from the SCN is only partly known. Is the information transferred via neurons? Is the information coded as pulse only?

In birds the SCN of the mammal is rep-

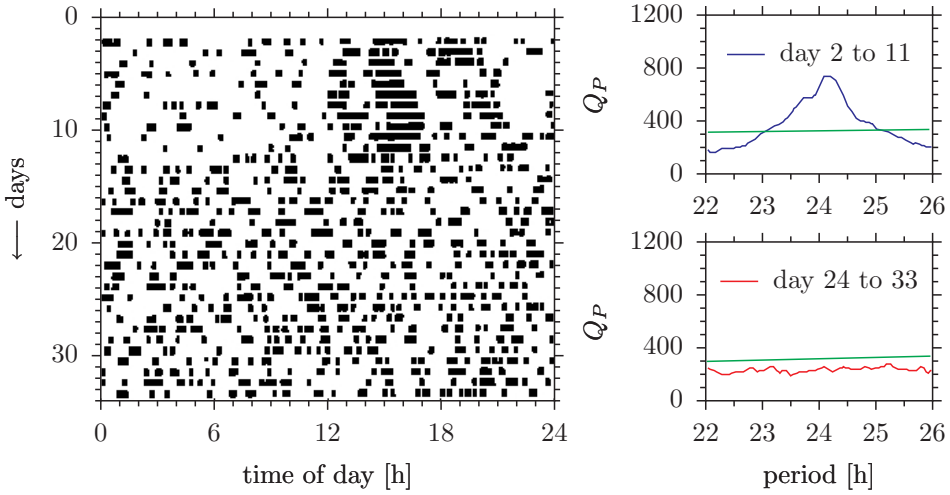

Figure 3.13: *The circadian rhythm of* locomotor activity *disappears after lesioning the suprachiasmatic nuclei (on day 11). Periodograms for day 2 to 11 at the top right, for day 24 to 33 below. After [1210]*

resented by the visual SCN (vSCN). According to a ring model the pineal and the vSCN function in birds as damped oscillators with a mutual phase relationship of 180°: The pineal is active during the night, the vSCN during the day. The eye possesses a rhythm which is driven by the visual system or functions as another oscillator ([1220].

3.6 Molecular mechanism of the circadian oscillators

A molecular model of the circadian clock of mammals is shown in figure 3.15. It consists of several clock genes which by feedback, by time delay and by interacting with transcription factors inhibit their own expression. Light synchronizes the oscillator by being absorbed by photoreceptors which send a signal to the clock genes.

3.7 Pineal organ and melatonin

The pineal organ is connected with the SCN and is synchronized by light which is absorbed in the retina (figure 3.16). The pineal organ forms a neuroendocrine feedback mechanism ([926]). It was assumed that in mammals -in contrast to birds and reptiles- the pineal has no influence on the circadian rhythm. However, melatonin does affect the SCN by feedback and modulates in this way the activity pattern and other circadian processes. The kind and mechanism of this feedback is unknown. Although pinealectomy in rats does not influence the circadian rhythm in the light-dark-cycle and in continuous *darkness*, the running wheel activity in pinealectomized rats is heavily disturbed in continuous *light*. Either the feedback from the pineal to the SCN modulates the light sensitivity of the SCN or the circadian output from the SCN ([215]).

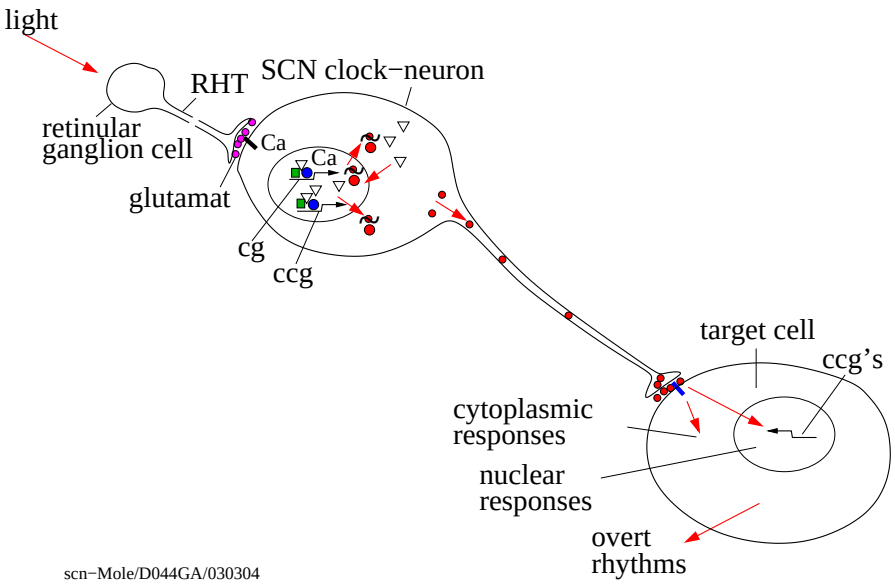

Figure 3.14: *Events between light reception, SCN clock neuron and target cell. Light is received in a retinular ganglion cells. The neurotransmitter glutamate (violet) is released as and reacts with receptor (black rectangle). Via a negative feedback loop with amplifying factors CLOCK (green) and BMAL (blue) the expression of mPer and mCry the clock gene (cg) is turned on. Ca^{2+} is also involved. Clock protein mRNA (red spheres with ~) is produced, leaves the nucleus and synthesizes clock protein (triangles) in the cytoplasm. It enters the nucleus, interacts with mPER and facilitates its translation by blocking CLOCK-(green) and BMAL-(blue) dependent transcription. As a result mRNA concentration decreases. With a lag negative acting complexes are inactivated and gene expression starts up again. The next round of negative and positive acting factors drives the rhythmic expression of clock-controlled genes (ccg´s). Its product, clock controlled proteins, provide information of time of day to the SCN neurons and via synaptic or paracrine signals to their target cells. Target specific circadian outputs via cytoplasmic responses or via nuclear responses affect secondary ccg´s. An example is the N-Acetyltransferase, which controls melatonin synthesis. After [583]*

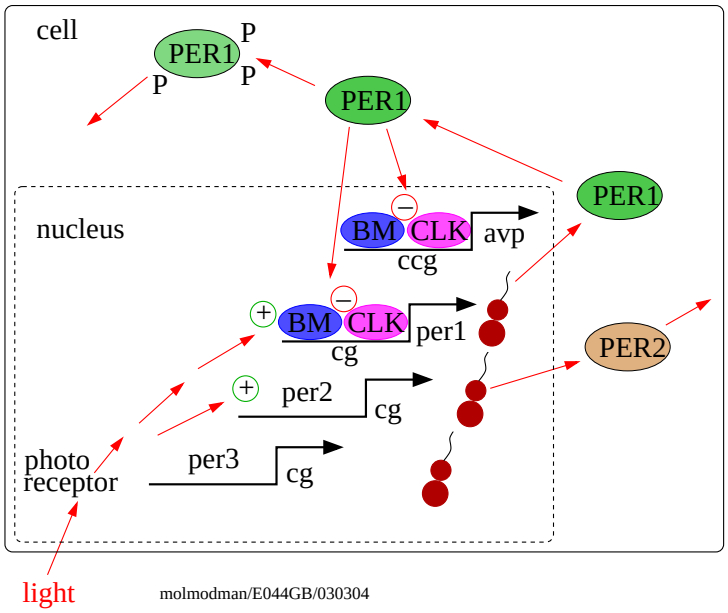

Figure 3.15: *Molecular model of the circadian clock of mammals: Clock genes mper1, mper2 and others (not shown) feed back with time delay and by interacting with transcription factors BMAL and CLK on their own transcription inhibiting it (per1, per2 and per3 are clock-genes cg, brown: mRNA). PER is decomposed by phosphorylation (P, brighter green color). Light synchronizes the oscillator by being absorbed by photoreceptors which send a signal to the clock genes. Clock controlled genes (ccg) such as the avp gene, which expresses AVP, are also inhibited by PER1. Nucleus dashed, cell solid rectangles. After [773], [338] and [1230]*

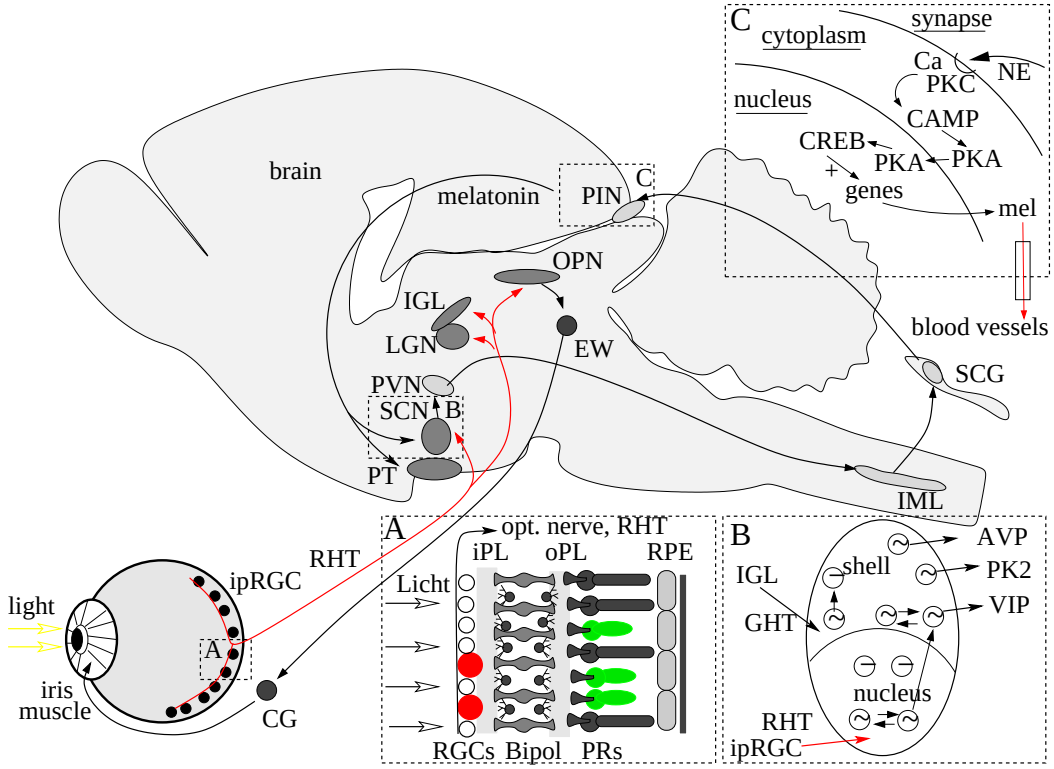

Figure 3.16: *Neuronal organization of circadian system in mammals. The suprachiasmatic nucleus SCN consists of many cellular oscillators which are organized and coupled with each other in a specific way. Light synchronizes the SCN directly via signals through the RHT (neurotransmitters are glutamate GLU and substance P SP) and indirectly via the intergeniculate leaflet IG and the geniculohypothalamic tract GHT (neurotransmitters are neuropeptide Y NPY and gamma-aminobutyric acid GABA). There is furthermore a serotonergic input into the SCN from the raphe nucleus (serotonin as neurotransmitter). Circadian signals influence melatonin secretion of the pineal organ via a multi-synaptic connection. It includes the superior cervical ganglion, the para-ventricular nucleus, the pre- and post-ganglionic fibers of the peripheric sympathetic nervous system (not shown). Melatonin is secreted into the blood system. It also feeds back (red arrow) to the SCN. The SCN controls furthermore a multitude of physiological and behavioral events, among them the hypothalamic temperature and locomotor activity. The latter feeds back to the SCN (red arrow). After [1580]*

Already in the thirties of the twenties century it was known that reproduction of different rodents is controlled by the day-length ([45], [104]). The significance of the pineal organ for the photoperiodic control was slowly clarified. First it was assumed, that melatonin is secreted by the pineal, in order to transfer the inhibiting effect of the short day on the gonadal system. The pineal was thought to be an antigonadotropic gland. Many experimental results spoke in favor of it. Later it was found that the pineal possessed also a progonadotropic effect, depending on the species ([620] in ferrets, [643] in the Djungarian hamster). Besides the reproductive system in the Djungarian hamster body weight, pelage coloration ([644], [428]) and body temperature regulation are under photoperiodic control ([1414]). This shows, that the pineal and its hormone melatonin transmits the photoperiodic signals of the environment to the neuroendocrine axis. These signals can be either stimulating or inhibiting ([648]).

Melatonin affects, as mentioned, not only the gonads and other physiological processes, but also the SCN. Here it inhibits the neural activity and shifts the phase of the circadian rhythm (figure 3.17).[5]

Melatonin plays also an immunoregulatory role ([247]). More on melatonin is found under special topics in section 20.8. Concerning the physiological effect of the photoperiod on reproduction of mammals see section 13.4. For a review on melatonin and its action as a photoperiodic signal see [1415] and the special edition of the Journal of Biological Rhythms ([280]).

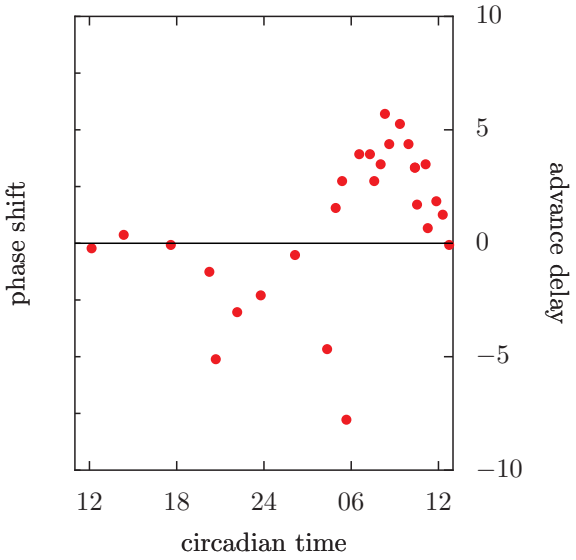

Figure 3.17: *Whether a melatonin pulse advances or delays a circadian rhythm in humans and how strong this phase shift is, depends on the time the pulse is applied (x-axis, circadian time). After [890]*

3.8 Feedbacks and masking

The locomotor activity is not only controlled in a circadian way, but it feeds back to the circadian oscillators in the SCN. In a hyperactive mutant (Wocko-mouse) the period is shortened ([1397]). It is known that serotonergic afferents of the raphe nucleus in the mid brain project to the circadian pacemaker in the SCN. If they are switched off by micro-injection of serotonergic inhibitors, without influencing the noradrenalin content and the neuropeptide Y-immunoreactivity, the amount of wheel running is not influenced. The animals are, however, synchronizable by two or more hours of daily running wheel activity. Serotonergic afferents are thus

[5] a melatonin agonist shifts the circadian rhythm of mice (and Syrian hamsters) as a function of phase ([1499])

needed for this kind of synchronization ([361], see figure 3.16).

The period length can be influenced, if the access to the running wheel is limited to a certain time of the cycle. The period is shorter, if the mice are allowed to run at the onset of the subjective night (at the onset of their activity period) and longer, if allowed to run at the end of the subjective night (at the end of their activity period) ([359]. This indicates, that the clock can be influenced by physiological and/or behavior-dependent factors.

If the amount of locomotor activity of mice is influenced, the sleep-wake-cycle and the activity rhythm can be modified. It is, however, not a basic change in the clock-mechanism and its coupling, but a matter of 'masking' ([1043], [358]).

3.9 Mutants of the circadian system in rodents

A mutant was discovered in Syrian hamsters by chance, the locomotor activity of which exhibits a period of 22 hours instead of 24 hours in the heterozygous and of 20 hours in the homozygous animals. This mutant was called tau (figure 3.18). The responsible gene is autosomal. The mutation is semi-dominant ([1198]). Unfortunately the genetics of the Syrian hamsters is poorly studied.

The genetics of **mice** are much better studied as that of the Syrian hamster. It was therefore a lucky circumstance when a mutant was found the period of which had changed. It was obtained by treating males with ENU[6]. The treatment renders the animals sterile, but after 12 to 16 weeks they become fertile again. They

[6] mutagenesis by N-ethyl-N-nitrosourea

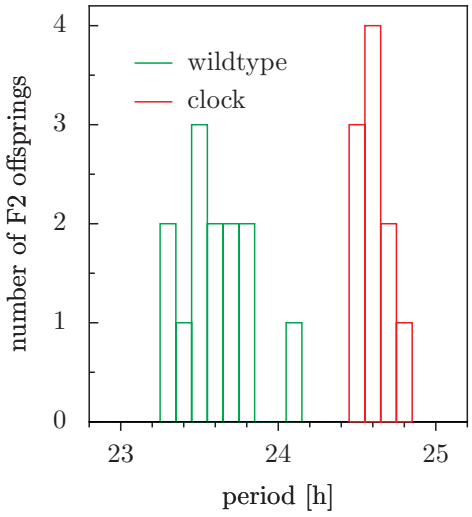

Figure 3.19: *The period length of the 'clock' mutant ('circadian locomotor output cycles kaput') of golden hamsters is lengthened by 1 hour in the heterozygous condition, and in the homozygous condition by 4 to 5 hours (in darkness, actograms at the top). After 5 to 15 cycles the animals become arrhythmic. They show now an ultradian rhythm of 6 to 9 hours. Crossing heterozygous animals with each other leads to two different groups in the F2 generation with a mean period around 23.5 and 24.6 hours, respectively. After [1517]*

were crossed with females and the first generation of progeny checked for mutants. It was expected that one among 1500 animals is mutated in a certain locus. And indeed a mutant was found (figure 3.19, [1517]).

The period length of this 'clock' mutant ('**c**ircadian **l**ocomotor **o**utput **c**ycles **k**aput') is in continuous darkness lengthened by 1 hour in the heterozygous condition, and in the homozygous condition by 4 to 5 hours. After 5 to 15 cycles the animals become arrhythmic. They show now an ultradian rhythm of 6 to 9 hours.

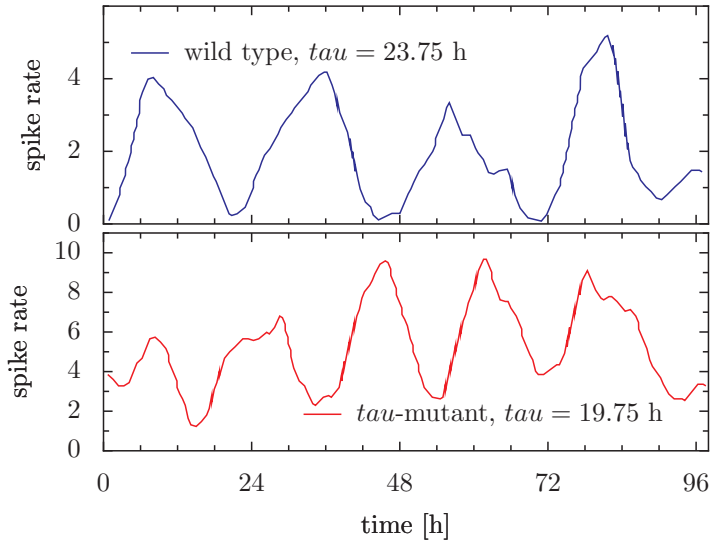

Figure 3.18: *tau-mutant of the Syrian hamster. The locomotor activity rhythm possess a period length of 20 hours in the homozygous condition (bottom), whereas the period length of the wild type is 24 hours (top). Heterocygous mutants show a period of 22 hours (not shown). After [1198].*

The development of the mutant animals is normal, the locomotor activity during the day relatively high. CLOCK is an essential gene for the circadian system ([774], [19]. It is positioned in the center of the fifth chromosome. The mutant is not causing a defective SCN.

The Clock-gene was cloned (cloning by rescue, [774]). It has a large transcription unit with about 100 000 base pairs and 24 exons. It codes for a so far unknown basic-helix-loop-helix-PAS domain protein. Over-expression of the Clock transgene shortens the period length ([19]). CLOCK is thus another example of a clock-protein with a PAS-domain (PERIOD of *Drosophila* is the other). This motif could represent a conserved property of the circadian clock mechanism during evolution ([774]).

A further mutant, Whl, was found. The mutation lies on the fourth chromosome. Its period length was longer as that of the original strain (24.20 versus 23.32 hours) ([1156]). This mutant has other abnormal properties such as hyperactivity, circular movements and a changed reaction toward light.

In another mutant mouse, which lacks a neuronal adhesion molecule with polysialic acid, the circadian rhythm is also changed ([1374]).

Not only mutants, but also strains can differ in their circadian periods ([650]). In darkness the periods for strain B6 were 23.8 h, for D2 23.7 hours and for C 23.6 hours. In continuous light the periods amounted to 25.1 hours for B6, 23.9 hours for D2, and 25.5 hours for C. It is assumed that polygenic inheritance is involved in this case ([972]). The two strains BALB/cByJ and C57BL/6J differ by 50 minutes in their free run period ([1349]).

The circadian rhythm of the activity of

BALB/c mice is very labile. Free run period and coherence of the running wheel rhythm changes spontaneously. The circadian system of this strain consists apparently of a population of weakly coupled oscillators ([1267].

It is to be expected that in the next future many new results will turn up in this field by using molecular biological procedures. A citation of [996]: *"Reverse genetics" (from gene to phenotype) with targeted gene transfer provides a powerful tool to dissect behavior and has been used successfully to study the effects of null mutations in genes implicated in the regulation of long-term potentiation and spatial learning in mice. In addition, "forward genetics" (from phenotype to gene) with high-efficiency mutagenesis in the mouse can uncover unknown genes and has been used to isolate a behavioral mutant of the circadian system. With the recent availability of high-density genetic maps and physical mapping resources, positional cloning of virtually any mutation is now feasible in the mouse. Together, these approaches permit a molecular analysis of both known and previously unknown genes regulating behavior.*

3.10 Other circadian rhythms

Besides the locomotor activity and body temperature rhythm other rhythms have been studied in rodents. The sleep-wake-rhythm can be recorded via the locomotor activity. More precise is an EEG. Only with an EEG it is possible to determine unambiguously whether an animal is asleep. Furthermore with an EEG the different sleep stages can be determined. An automatic recording unit and special evaluation methods were developed ([486]).

'Disk shedding' is also under circadian control. The rods (night vision) and cones (day vision) of the vertebrate eye are not, as other cells of the body, renewed during the course of time. Instead the disks in the tip of the outer segment (arranged like a stack of coins) are shed and renewed at the base of the cones. This internal renewal occurs at times when the photoreceptors are not used. Rods are therefore regenerated in the morning, and cones in the evening.

Male house mice develop about 18 to 20 days after the coitus (ejaculation) father behavior toward their own offspring. 50 to 60 days later it disappears again and pubs are killed by them. This behavior is synchronized with the reproductive cycle of the females. It functions, however, also without social keys and without changes in the hypophysis- and gonadal hormones. If the animals are kept in a 22-hour day (11 hours light, 11 hours darkness) or in a 27 hour day (13.5 hours light, 12.5 hours darkness), this behavior occurs after the same number of cycles, but not after the same number of days; it is therefore controlled by a circadian clock. The physiological basis of this behavior is unknown ([1152]).

3.11 Photoperiodism in mice?

Whereas many rodents show a seasonal pattern of propagation and use thereby photoperiodic time cues (for instance *Peromyscus maniculatus* ([1066]), although even here not all animals of a population react photoperiodically ([109], [1277])[7],

[7] Animals of a population of *Peromyscus maniculatus* differ in their reaction to short day: Some animals reduce their gonads completely, others partly, and others not at all. It was assumed that the pattern or amplitude of the melatonin-rhythm was different. It was found out, however, that the energy demand was different. The varying reaction to the photoperiodic treatment was therefore not brought about by events in the

mice do not seem to possess a photoperiodic reaction of propagation. They are, however, able to use photoperiodic informations (such as control of the fur density). But this feature is not coupled with reproduction, although recent observations have shown that the size of the ovary and the mass of the uterus of female mice react to the photoperiod ([1064]).

The tropical mouse *Zygodontomys brevicauda* does not exhibit a photoperiodic behavior ([593]).

3.12 *Mus booduga*

The Indian field mouse *Mus booduga* was studied in Madurai in southern India by Chandrashekaran and coworker ([1370]). The locomotor activity is controlled by a circadian clock. Light synchronizes this rhythm. Single light pulses given at different phases during continuous darkness result in a PRC with delays and advances of the rhythm depending on phase ([1368]). Near UV light does also phase shift ([1371]).

In spite of the fact, that twilight amounts to 15 to 20 minutes only at the latitude of Madurai, natural twilight has a stronger entraining effect as compared to abrupt light-dark changes ([1370]).

The precision of the clock depends on the period length of the rhythm. Using onset of activity, the highest precision is reached by animals with a period length of 23.8 hours, whereas longer and shorter periods reduce precision ([1369]).

Melatonin phase shifts the rhythm. It accelerates adjustment of the rhythm to advancing light dark cycles (corresponds to eastward flight), but combats adjustment of the rhythm to delaying light dark cycles (corresponds to westward flights) ([1372]).

pineal, but by consecutive events. The environmental temperature had no effect on it ([1277]).

4 *Lingulodinium*: Circadian rhythms

In the unicellular alga Lingulodinium *several circadian rhythms have been studied such as one in bioluminescence, in the aggregation of cells, in cell division, and in photosynthesis. A number of enzymes have been shown to be under the control of the circadian clock. Light synchronizes these rhythms. Certain substances are able to influence the rhythms. Other dinoflagellates such as* Pyrocystis noctiluca *and* Pyrocystis lunula *display also circadian rhythms. For instance, the movement of chloroplasts and their ultrastructure fluctuate in these species in a circadian way. It will be shown how bioluminescence is recorded. Its biochemical control is mentioned and its significance for the algae pointed out.*

You might have experienced already the unforgettable impression of diving in a cloud of millionth of tiny luminous particles when jumping at night into the water of the Mediterranean sea or the pacific ocean or some other warm sea. The reason for this spectacular firework is the bioluminescence of a number of different dinoflagellates. One of it is *Lingulodinium*[1], a 1/20 mm sized unicellular of the Pacific ocean.

These algae possess a cellulose skeleton with an equatorial and a longitudinal ridge (figure 4.1). In each ridge a flagellum is positioned. For photosynthesis chromatophors are present. Under certain conditions mass production might occur

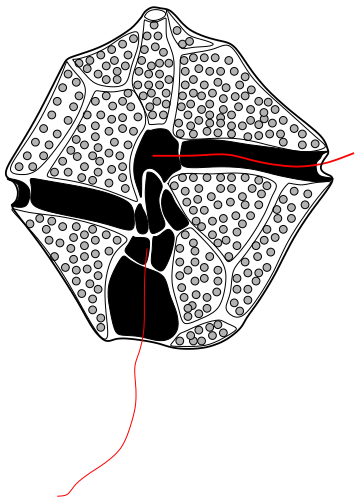

Figure 4.1: Lingulodinium polyedrum *cell with cellulose case, an equatorial and a latitudinal rim, in each of which a flagellum is located. Ventral view. Diameter 40 µm. After [1340] and electron microscopic picture ([577])*

[1]The marine alga *Lingulodinium* (older name *Gonyaulax*) belongs to the division of *Dinophyta*, and here to the class of *Dinophyceae* and to the order of *Peridiniales*.

Figure 4.2: *Aerial view of a 'red tide' during the day: The density of the dinoflagellates is so high that the population is visible due to the red fluorescence of the chlorophyll. After Taylor in [576]*

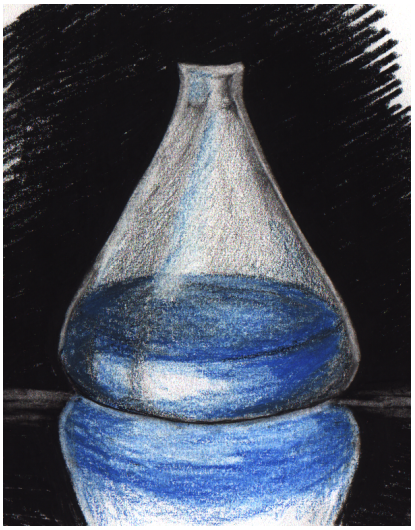

Figure 4.3: *The bioluminescence of a* Lingulodinium polyedrum *culture was photographed briefly after shaking the Erlenmeyer-flask. Painted from Mareike Förster after Taylor in [576]*

and the population is visible also during the day due to the red fluorescence of the chlorophyll ('red tides') (figure 4.2, [15]).

The bioluminescence can also be observed under laboratory conditions. The cultures can be reared easily in flasks[2] (figure 4.3). If kept in a 12:12 hour light-dark-cycle and observed at different times of the day[3] the bioluminescence is observable during the night only. But this rhythm pertains also under conditions of constant light. It is therefore controlled by an internal clock.

This rhythm has been studied intensively in different laboratories and belongs to one of the best known (reviews [1442], [574], [1257]). The bioluminescence can be recorded for extended times automatically and in many vials simultaneously.

More on the phenomenon of bioluminescence in *Lingulodinium*, its rhythmic control, how to influence the rhythm and on which mechanisms it is based, in the following. A video movie is available on bioluminescence and circadian rhythmicity in dinoflagellates ([76]).

4.1 Rhythmic bioluminescence

4.1.1 Recording equipment for the measurement of bioluminescence

In the laboratory the bioluminescence can be recorded automatically in the following way (figure 4.4): A sledge with a photomultiplier is positioned underneath one of

[2] use 1 liter Fernbach flask with F/2 nutrient medium with soil extract without silicate. Light-dark-cycle is 12:12h, light intensity 130μEinstein/m^{2}sec (1μEinstein corresponds roughly to 60 lux).

[3] during the light period one has to switch off the light for a while and observe the cultures for bioluminescence, or use automatic recording devices as described later

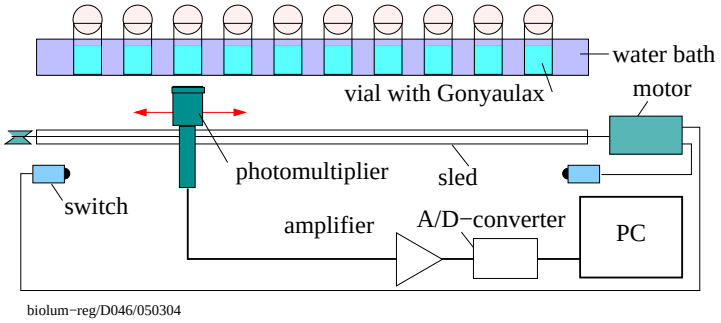

Figure 4.4: *Recording of the bioluminescence of* Lingulodinium polyedrum *using the 'Taylortron': A photo-multiplier is positioned on a sled with a stepper motor underneath a row of glass vials containing cultures of algae. After the last vial a switch returns the photo-multiplier in its starting position again. In this way the amount of emitted light of each culture can be recorded in predetermined periods of time for several days in continuous light. During the measurement the environmental light is shut off from this particular vial. The analog signals are amplified, digitized and stored on a computer ([344], [158])*

several glass vials containing 10 ml of a culture of algae ($5 * 10^3$ cells/ml). During the measurement (17.8 seconds/sample) the environmental light (0.5 to 14 lux is optimal) is switched off. Than the next culture vial is tested for bioluminescence, until all $2 * 30$ vials (including a light standard) are measured. After 20 minutes the recording starts again at the first vial. The signal is amplified and recorded via a computer. With the help of a special program the period length, the amplitude of the oscillation and the phase shift of the rhythm is determined. By using special measures the glow- and flash rhythm can be distinguished and recorded separately. Bioluminescence was recorded also in single cells ([828]).

4.1.2 Flash rhythm and glow rhythm

The bioluminescence of *Lingulodinium* consists of two phenomena: a series of flashes caused by a mechanical or a chemical disturbance and a much weaker glow which is observable in an undisturbed culture (figure 4.5). The bioluminescence of the flash rhythm is strongest in the middle of the dark period and lasts a few hours only each day. A flash lasts 100 ms. During this time about 10^7 to 10^{10} light quanta per cell are emitted. The glow rhythm however has its strongest bioluminescence toward the end of the dark period. Properties and differences between flash- and glow rhythm are compiled in table 4.1.

The period length amounts to 24.4 hours at 1200 lux continuous light. At 3800 lux the period is 22.8 hours and the rhythm dampens. At an intensity beyond 10000 lux the bioluminescence rhythm disappears. In continuous darkness the period is 23.0 to 24.4 hours and the rhythm dampens.

The bioluminescence rhythm is, as is the rule for circadian rhythms, relatively independent of the environmental temperature (figure 4.6, [1444]). At higher temperatures the clock of *Lingulodinium* runs somewhat more slowly, that is, the Q_{10} (see Glossary) is 0.85 ([581]). The

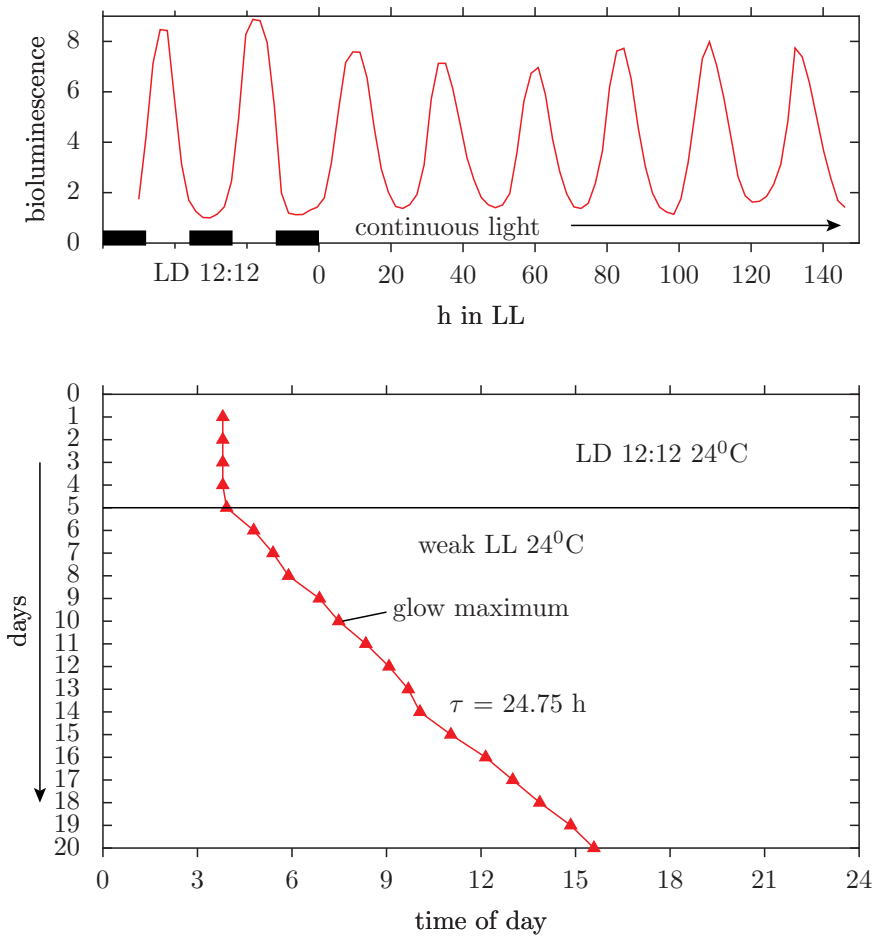

Figure 4.5: *Upper curve: glow rhythm (y-axis: bioluminescence) of a culture of* Lingulodinium polyedrum *in a 12:12 hour light-dark-cycle (up to hour 0) and afterward in weak continuous light. Bottom curve: glow rhythm of a culture of* Lingulodinium polyedrum *at a constant temperature of* 24^0*C in 12:12 hours light-dark change (up to the fifth day) and afterward in weak continuous light. Only the maximum of the light intensity of the glow rhythm is indicated by a triangle for each day. The period length of the rhythm is in the light-dark cycle 24 hours (synchronized) and in continuous light 24.75 hours ('free run'). After [575]*

Table 4.1: *Differences between flash- and glow rhythm of* Lingulodinium polyedrum

event	flash rhythm	glow rhythm
induction	mechanical and chemical stimulation	spontaneous
duration	100 ms	continuously
maximum	middle of subjective night	end of subjective night
period	LL: 22. 8h, DD: 23-24. 4h	22. 5
synchronization	6:6 to 16:16	6:6 to 16:16
PRC	yes	yes
Temp. comp.	$Q_{10} = 0.85$	$Q_{10} = 0.85$

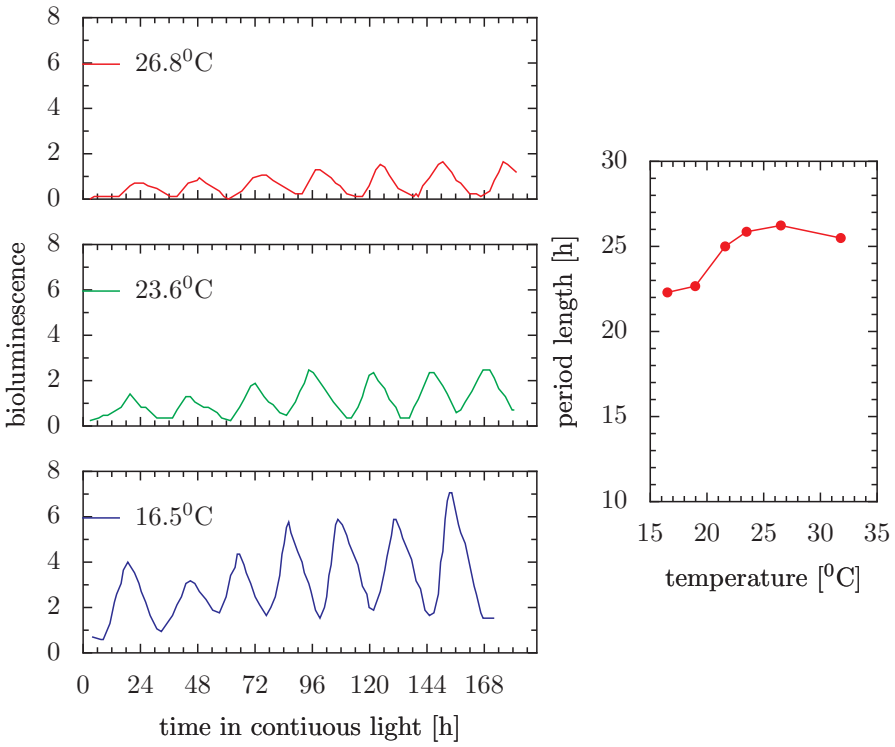

Figure 4.6: *The period length of the flash rhythm of* Lingulodinium polyedrum *is only slightly dependent on the temperature of the sea water ('temperature-compensated'): The time course of bioluminescence (in weak continuous light) is plotted at different temperatures (upper left curve: 26.8°, middle left curve: 23.6°, lower left curve: 16.5°C). y-axis: Amount of emitted light. Right curve: Period length (in hours) of the glow rhythm of the bioluminescence of* Lingulodinium *as a function of the temperature of the medium. After [581]*

temperature compensation is explainable by two chemical reactions with similar temperature-dependencies, in which one reaction product inhibits the other reaction (figure 16.9). The clock is in this way buffered against changes in the environmental temperature.

4.1.3 Precision and communication

The bioluminescence rhythm is very precise. For the population it might be up to 2 minutes per day (0.015%) (figure 4.7). For the individual cell the variability of the period length amounts to 18 minutes per day (1.36%) ([1095], [1038]). Under constant conditions the rhythm of bioluminescence stays on for a long time; the synchrony, however, decreases and therefore the maxima become broader.

That the rhythm does not damp out earlier can be explained in two ways: Either the period lengths of the clocks which drive the rhythm are very similar. In this case it takes quite some time until the rhythm of the population damps out. Or the cells are able to communicate with each other and to synchronize each other. Against such a chemical communication speak experiments, in which cultures in different phases were mixed with each other. Their rhythm after mixing was as one would expect if there were no influences ([1433]). However, if the medium was not changed, a mutual synchronization was observed after nine days ([157], [579]). Under natural conditions in the ocean this would of course never happen, since the medium is constantly exchanged by the water movements.

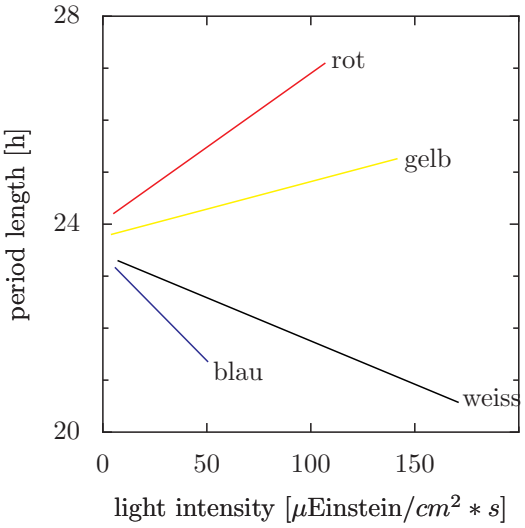

Figure 4.8: *Dependency of period length (y-axis) of the glow rhythm of the bioluminescence of* Lingulodinium polyedrum *on the wave length (red, yellow, blue, white) and on the intensity of the continuous light (x-axis). After [1250]*

4.1.4 Synchronization by light and photoreceptors

As in most other organisms light is the strongest Zeitgeber for synchronizing the *Lingulodinium* clock. Light affects furthermore the period length of the bioluminescence rhythm. It depends on the light quality and the amount of light: Under continuous red light it is longer than 24 hours and will increase further under higher intensity. Under continuous blue light it is shorter and will further shorten under higher intensities (figure 4.8). Furthermore in red light the flash is stronger than the glow and both kinds of luminescence are stronger as compared to white light.

Light pulses given during weak background light shift the rhythm. A phase response curve plots the reactions. It

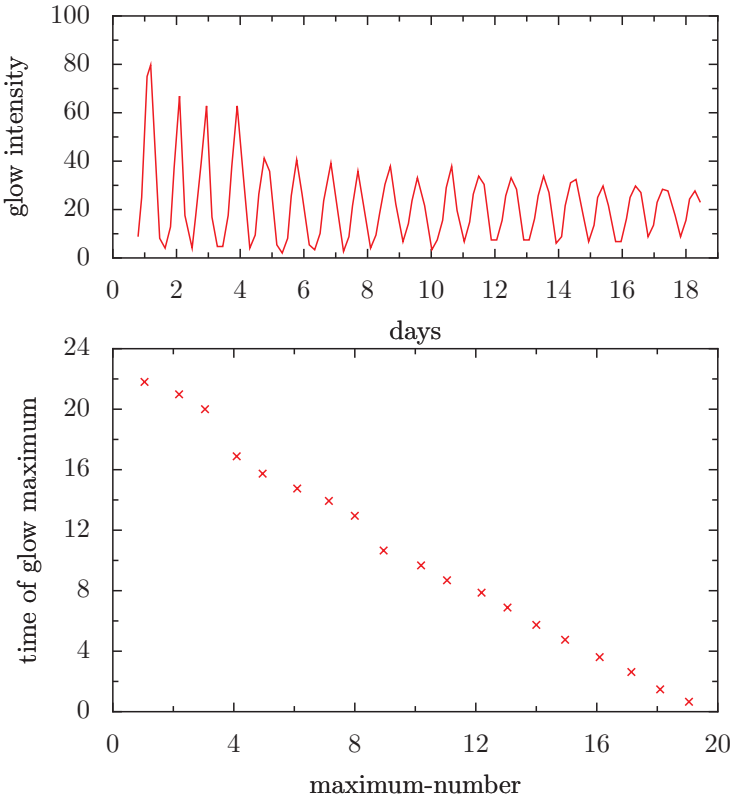

Figure 4.7: *The circadian glow rhythm of bioluminescence of* Lingulodinium polyedrum *was determined in a culture, which was first kept in a 12:12 hour light-dark cycle and a constant temperature of 19°C. At time 0 (x-axis) the algae were transferred into weak continuous light (upper part of the figure). The precision of this rhythm is demonstrated in the lower part of the figure. Here the clock time of the bioluminescence-maxima are plotted against the number of the maxima. The precision is even higher, if the maxima of the bioluminescence are connected with three straight lines (the cause of the two phase shifts is unknown). According to [1038]*

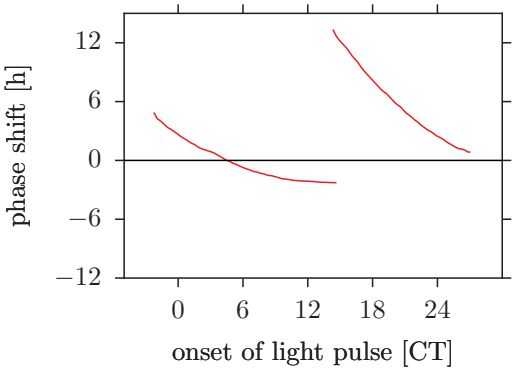

Figure 4.9: *Phase response curve for the flash rhythm of* Lingulodinium polyedrum *toward a 4-hour blue light pulse of 175 µEinstein/m²sec presented to cells in red light at different phases. The phase shift by the light pulse (earlier as the control: above the zero line, later as the control: below the zero line) depends on the phase (x-axis), at which the light pulse is given. CT: circadian time. After [707]*

is asymmetric with small delays and stronger advances (figure 4.9). For day-active organisms this makes sense. They can better adapt to the varying light periods in the course of the year. In contrast to the visible light, UV-light shifts the rhythm of the bioluminescence to earlier times only (advances the rhythm, see [1438]).

The action spectrum shows maxima in the blue (475 nm) and red (650 nm) spectral region ([582]). This could indicate chlorophyll as the responsible photoreceptor. However, this was excluded experimentally. Neither does phytochrome participate in synchronizing. Apparently two different photoreceptors are involved, but their nature is unknown so far. The phase shift by light pulses can be canceled by substances which inhibit respiration of the mitochondria or which serve as H^+-ionophores in membranes of mitochondria and chloroplasts (azide, DNP, rotenon, CN).

4.1.5 Chemical control of bioluminescence

If we want to understand the rhythmic control of the bioluminescence, it is certainly of help to know how this phenomenon is brought about both physiologically and metabolically. Hasting's group has worked a great deal on this problem too, not only on the rhythmic control of it. But there are still questions to be answered.

Bioluminescence occurs in special spherical organelles, the scintillons. Their diameter is 0.5μ, their molecular weight 10^9. During the light period there are only about 40, during the dark period about 400 scintillons per cell ([461], [704]). Scintillons have a dense matrix and lie in the neighborhood of the cell membrane (figure 4.10). They protrude into the vacuole like pockets and are connected with the cytoskeleton. With imaging amplification and video-microscopy a blue bioluminescence (470 nm maximum) can be recognized. The scintillons can be established with immunocytological tests using gold particles and antibodies for luciferase. Scintillon-extracts do also flash, if transferred from a pH of 8 into one of pH 6. Scintillons are also connected with the cytoskeleton.

As in all other bioluminescence reactions the luminescence of *Lingulodinium*-cells consists of a reaction of a substrate, luciferin with an enzyme, luciferase.[4]

[4] Luciferase has at a pH of 8 a molecular weight of 140 kDa and is a dimer (each 70 kDa). At pH 6 the molecular weight is 35000 to 40000. Its maximal activity is at a pH of 6.4. A 4.1 kb mRNA

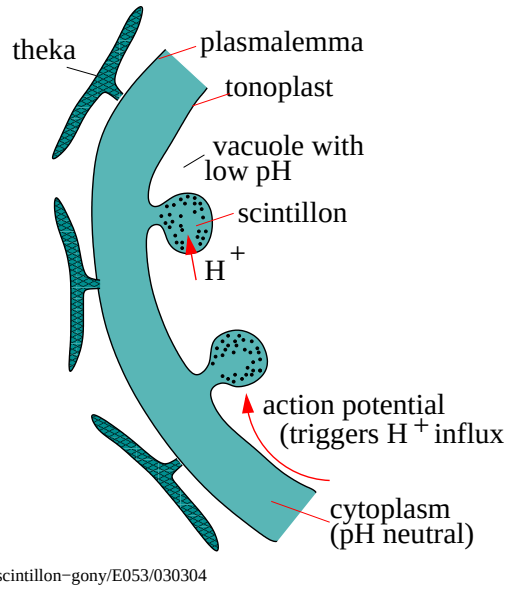

Figure 4.10: *Scintillons are organelles for the production of bioluminescence in* Lingulodinium polyedrum. *During the night a cell contains about 400, during the day only about 40 scintillons. They protrude as pockets of the tonoplast from the cytoplasm into the vacuole. Diameter about 0.5 µm. Shaking the culture or other stiumulations of the cells induce an action potential. This triggers an H^+-flux from the acid vacuole into the less acid scintillon. As a result bioluminescence occurs (see figure 4.13). Theka: Armored plate of the casing. After [1086], [580]*

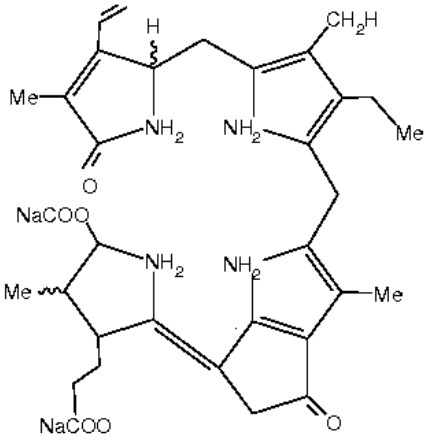

Figure 4.11: *Tetrapyrrole-structure of luciferin from* Lingulodinium polyedrum. *Me: Methyl group. After [1052]*

There are $2.7 * 10^{12}$ luciferase-molecules per cell. The luciferin is in the case of *Lingulodinium* a tetrapyrrole, which is a small molecule (molecular weight<1000) (figure 4.11). It is heat stable, whereas the luciferase is not. By oxidation the luciferin is converted to the singlet condition by emitting light. The scintillons contain furthermore a luciferin binding protein LBP. It sequesters luciferin, if the pH is 7.5 (the normal pH of the cytoplasm) or larger. At a pH of 6.5 or lower the configuration of the LBP changes, the luciferin is released and reacts with O_2 through luciferase. Lower pH activates furthermore the luciferase.

$LBP - LH_2\,(pH7.5) \Rightarrow (H^+)\,LBP + LH_2\,(pH6) \Rightarrow (O_2, Lfase) \Rightarrow h\nu + L \Rightarrow O + H_2O$

A circadian clock controls the *translation* of the luciferin-binding protein, the luciferin (LH_2) and the luciferase ([1037])[5].

produces the luciferase. The cDNA was cloned. It contains no introns and differs from all other luciferases known so far.

[5]The mRNA for LBP (2.5kb sized) is constant and equally translatable in an in vitro system ex-

The molecular basis of it has been studied by [1019] and her group.

This translational control contrasts with the circadian control in the cyanobacterium *Synechococcus* and the Crucifers *Arabidopsis*, in which *transcription* is under circadian control.

Translation controlled regulatory clock-proteins (CP1, CP2, CP3) were proposed to compose the circadian mechanism (figure 4.12). CP1 cancels the repression of the synthesis of CP2 by interacting with the repressor of the mRNA-2. CP2 interacts with the regulatory region of mRNA-3, which is responsible for the synthesis of CP3. In this way a cascade takes place, in which each protein inhibits its own synthesis, until the cycle is finished.

The luciferase concentration runs parallel to the bioluminescence of intact cells. Around midnight it is about 10 times higher as it is around noon. The maximum is 6 hours after onset of darkness. In continuous light this rhythm continues with a low amplitude. The rhythmic course of the luciferase-activity could be due to modification of the enzyme by phosphorylation, methylation, activation or inhibition. Alternatively the amount of enzyme could change in a circadian way. That was indeed found ([342], [706]). Therefore either synthesis or destruction or both is oscillating in a circadian way.

A mechanical or a chemical (Ca^{2+}, NH_4, K^+, H^+) stimulation leads to an action potential. This reaches via the tonoplast the scintillons and depolarizes them. As a result H^+-ions enter the scintillons. Due to the quick pH change (from pH8 to 6) the LBP releases luciferin which reacts with luciferase. Light is emitted (figure 4.13). After the stimulation luciferin is bound again to the LBP and a new stimulation can take place ([437]). The circadian fluctuations of the reactants are perhaps due to the destruction and re-synthesis of the scintillons during each cycle. The spontaneous bioluminescence (glow rhythm) occurs possibly during the destruction of the scintillons. How this occurs is not yet clarified. Either the scintillons are tied up and decomposed in the vacuole, or they are emptied into the cytoplasm. The second possibility is more likely. The scintillons are dismantled in the early morning.

tracted from cells harvested at different phases of the cycle. The rhythm is therefore not based on a varying transcription. Instead it is controlled by translation. Accordingly the circadian rhythm of bioluminescence can be influenced by translational inhibitors, but not by transcriptional inhibitors. A clock-controlled trans-acting factor (a dimer?) might play a role in the LBP synthesis.

4.1.6 Circadian control of bioluminescence, mechanism of the clock

What about the circadian control of the bioluminescence, after having seen how the biochemical machinery works? The circadian clock controls periodically the synthesis and perhaps the destruction of luciferin, luciferase and LBP (figure 4.14). The *activity* of the luciferase and the degree of phosphorylation however stays constant.

Protein synthesis seems to be involved in the mechanism of the bioluminescence-rhythm, since inhibitors of protein synthesis (cycloheximid, puromycin, anisomycin) influence the period ([1457]). Cycloheximid pulses shift the rhythm of bioluminescence as a function of the phase of the oscillator. Protein-synthesis affects therefore the clock ([1338], but see also [1474]). Probably enzymes are involved with short half life times (0.5 to 1 hours) which limit metabolic rates, as is the case in other circadian systems (NATF

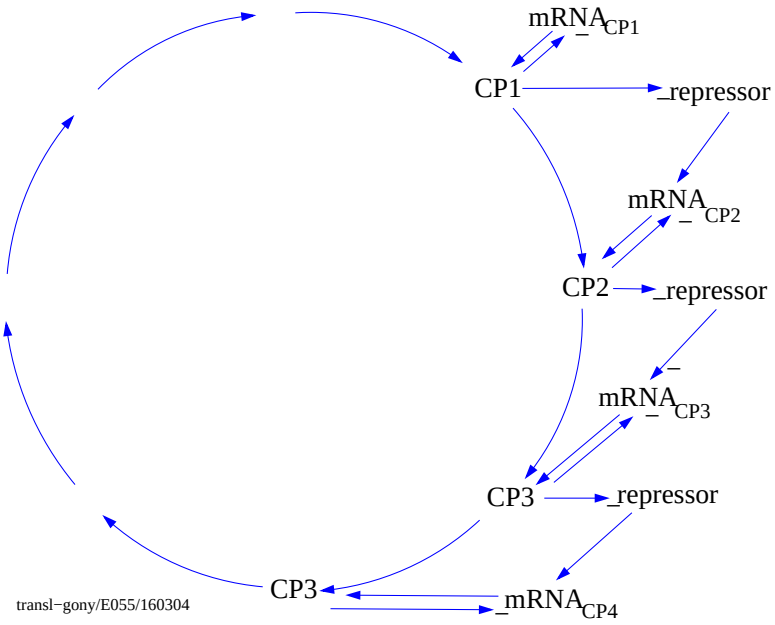

Figure 4.12: *Translational clock of* Lingulodinium polyedrum*: A cascade of clock proteins (CP's) forms the circadian oscillation. Each protein inhibits its own synthesis (since the synthesis should be short). The CP's are parts of the clock. From these proteins others have to be differentiated which are controlled by the clock. After [1038]*

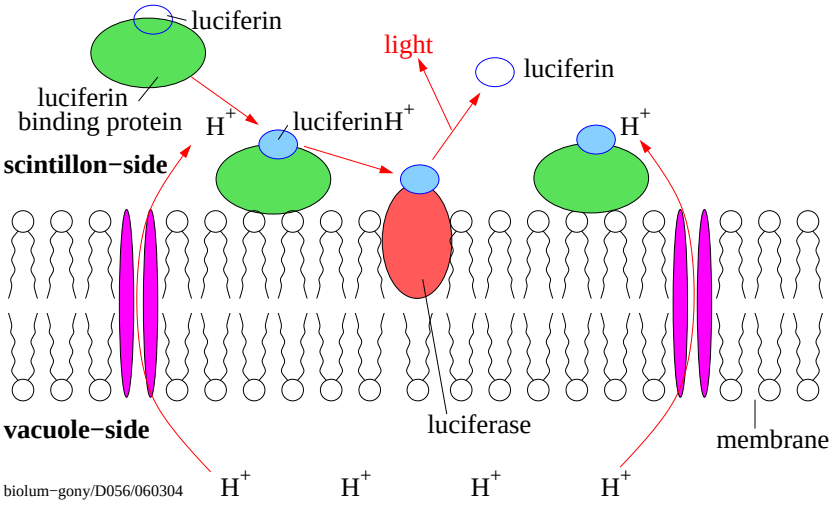

Figure 4.13: *Mechanism of light-production in* Lingulodinium polyedrum *in the scintillon. A small part of the scintillon-membrane is shown (vacuole would be below the membrane, the interior of the scintillon on top). If H^+-ions enter (e.g. as the result of shaking) the interior of the scintillon (left), it will turn more acid. The luciferin which was bound by the luciferin-binding protein becomes freed and will be oxidized by luciferase. During this reaction light is emitted. After [344]*

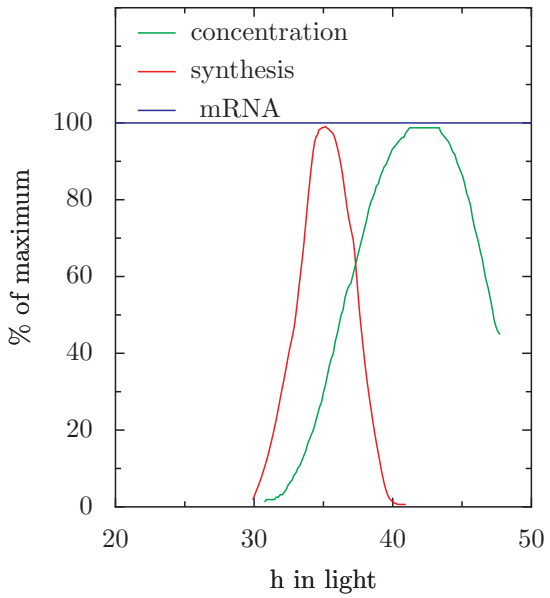

Figure 4.14: *The luciferin-binding-protein of* Lingulodinium polyedrum *is synthesized in a circadian rhythm (green curve, maximum of synthesis about 35 hours after onset of the continuous light following a 12:12 hour light-dark-cycle). The LBP protein reaches after 43 hours a maximum of its concentration (red curve). Afterward it is decomposed. The mRNA which is responsible for the LBP-synthesis is always present and active (blue curve). The circadian control of the LBP-synthesis occurs therefore on the translational level. After [1038]*

in the pineal, tyrosine aminotransferase in the liver, β- hydroxy-β-methylglytaryl-CoA reductase). Other enzymes have a half life time of several days.

Using anisomycin, arrhythmia could be induced, even at the level of the single cell ([1457] and figure 4.15). The oscillating system seems to consist of two state variables. One is closely connected with the protein synthesis on 80s ribosomes, and the other is influenced by light (and temperature?). Protein synthesis plays a role for the circadian rhythm also in other organisms: *Aplysia* ([687]), *Acetabularia* ([1352]).

Details of the circadian control of bioluminescence are still lacking. But several observations suggest that two clocks are involved. For instance, phase shifts by light pulses can influence the flash- and glow rhythm differently. Although the same luciferin and the same luciferase is used, the responsible reactions proceed differently. They probably occur in different compartments. Under certain conditions the period length of the glow- and flash rhythm differ (23.8 versus 23.6h) and, as a consequence, the phase relationship between both is changing (figure 4.16, [633]). The optimal light intensity for the two bioluminescence rhythms differs ($6\mu\, Einstein/cm^2 sec$ for the flash rhythm, $90\mu\, Einstein/cm^2 sec$ for the glow rhythm). The temperature influences the flash- and the glow rhythm differently, which speaks also in favor of two clocks ([633], [1035]). Finally, the bioluminescence rhythm and the aggregation rhythm can also possess different period lengths ([1254]). Thus there is substantial evidence for two clocks controlling the two different bioluminescences.

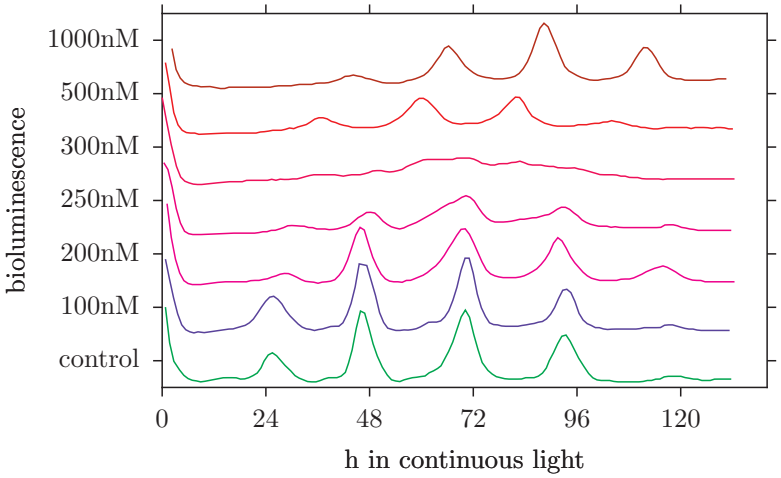

Figure 4.15: *Induction of arrhythmia in* Lingulodinium polyedrum *by an anisomycin-pulse of one hour duration. It was given 12 hours after onset of the continuous light treatment (this time is critical). The concentration of anisomycin was varied between 100 and 1000 nM (y-axis, next to the sample number). At a critical concentration of 300 nM the bioluminescence-rhythms disappears. After [1457]*

4.1.7 Significance of bioluminescence

What is the significance of the bioluminescence in *Lingulodinium* and other dinoflagellates? Among organisms bioluminescence is often observed (see also under special topics in chapter 20 on page 434). Different purposes are the cause of the usage of bioluminescence: Male and female animals can find and recognize the mates (glow worms), swarms can form, territories be marked. Fishes can illuminate the view field and attract bite (*Anomalops* in Japan). Bioluminescence can serve also as a protective measure. Enemies can be deterred, animals can use it for camouflage or distract enemies from the anterior end of the body (worms).

But why does *Lingulodinium* show bioluminescence, and why does it glow and flash? Bioluminescence might just be a metabolic byproduct. To get rid of electrons in the absence or shortage of an appropriate acceptor light can be emitted. For us bioluminescence is spectacular, for the alga just a way of disposing electrons. Other explanations have been offered, why they show bioluminescence. Thus, fishes swimming into a swarm of *Lingulodinium* and inducing a sudden bioluminescing could be frightened and therefore not feed the algae. The bioluminescence could also serve to synchronize the individual cells in a population. This is, however, unlikely, as experiments about mutual synchronization have shown. Further hypotheses have been proposed for the presence of bioluminescence, but none is very satisfying and none has been tested experimentally so far.

Another open question is, why the scintillons and their machinery are of such a short lifespan. Is it not a waste of energy to re-synthesize it daily? However, for an alga with plenty of energy available

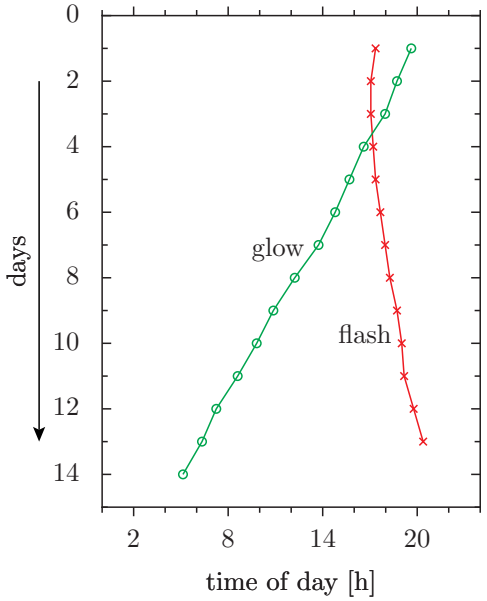

Figure 4.16: *Different period lengths in the flash- and glow rhythm of* Lingulodinium polyedrum *are found in continuous light at* 21^0C. *The daily maxima of the glow rhythm (open circles) and of the flash rhythm (dots) were plotted beneath each other and connected with one line. The period length of the glow rhythm amounts to 22.9, the one of the flash rhythm to 24.2 hours. After [633]*

it might be of more advantage to rebuild these organelles and use the nitrogen of the scintillon proteins for other enzymes because nitrogen is the limiting factor in this alga. To use a somewhat far stretched comparison: For us energy is more costly than water, but for an inhabitant of the oil producing countries it is the water which is more costly. Thus is makes sense for organisms with enough energy available, but limited nitrogen supply to safe nitrogen by breaking down enzymes which are not used at certain times. An enzyme with a short half life time (2-3 hours) serves this purpose better as one with a long life time.

4.2 Rhythms of aggregation, phototaxis, vertical migration and mobility

In *Lingulodinium* cultures swarm formation can be observed often (figure 4.17 and [1248]). This behavior is a population rhythm. It is observable under constant conditions for three weeks before becoming desynchronized. During the day the population stays close to the surface. It is not yet known which individual parameters (mobility, preferred direction of swimming, floating, differences in the microenvironment due to photosynthesis) lead to the swarming behavior. Perhaps the red tides of the phytoplankton is based on this phenomenon. Chemical signals do not seem to play a role.

The aggregation rhythm changes its period length with light intensity. However, the change depends on the wavelength of the light. In red light the period increases with increasing light intensity, in blue light it decreases (figure 4.18). It is assumed that two different photoreceptors

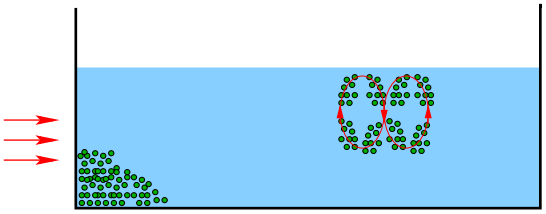

Figure 4.17: *Swarm formation in* Lingulodinium polyedrum *in a Petri dish, which was illuminated from the side with light of 120* $\mu E/m^2 sec$. *During the day aggregations are formed at the surface of the sea water, in the center of which the cells sink down and swim upward at the periphery. During the night the cells settle at the side which was originally closest to the light. After [1248]*

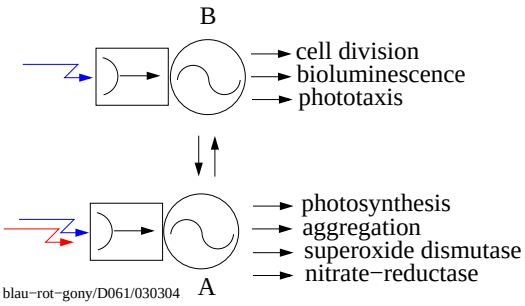

Figure 4.18: *Circadian system of* Lingulodinium *is thought to consist of an A- and a B-oscillator. The A-oscillator (below) controls among other properties the aggregation of the cells, whereas the B-oscillator controls besides cell division and phototaxis the bioluminescence. Blue light synchronizes the B-oscillator, blue and red light the A-oscillator. After [1035]*

and perhaps also two clocks are involved ([1250, 1254, 1036]). In favor of this interpretation is that light pulses influence the bioluminescence rhythm on the one hand and the aggregation rhythm on the other hand differently. The B-oscillator, which controls bioluminescence, reacts to blue light, the A-oscillator, which controls aggregation, reacts to blue and red light. In the green light (550 nm) the cells are blind ([1035]).

In *Gyrodinium dorsum* and *Gymnodinium splendens,* two other dinoflagellates, a diurnal rhythm in phototaxis was observed ([1442]). Its maximum of sensitivity is at a wavelength of around 450 nm. The rhythmic vertical migration (with a minimum before the light-off-time-point) can not be explained solely by phototaxis. Both rhythms are perhaps circadian.

In *Peridinium gregarium* a rhythm of mobility was found. During the night the cells become motionless and sink to the bottom. In *Lingulodinium* this might happen also in the light-dark-cycle, but under continuous light no rhythm is found.

4.3 Chloroplast rhythms

If one could use isolated chloroplasts which show still a circadian rhythm, it would be a nice minimal system and handy, because they are of large size and they can be manipulated in diverse ways. This is not yet possible, unfortunately, but in vivo several circadian changes in chloroplasts were observed. They are partly found in dinoflagellates other than *Lingulodinium*.

For instance, the number of chloroplasts, their form and ultrastructure change in a circadian way. In *Pyrocystis noctiluca* circadian movements of chloroplasts exist ([565] and figure 4.19). Furthermore, during the night the chloroplasts are contracted in the center of the cell, during the day however spread out. In *Pyrocystis lunula* the chloroplasts are also changing in a circadian way: However, here they retract during the night into the horns of the cell, whereas during

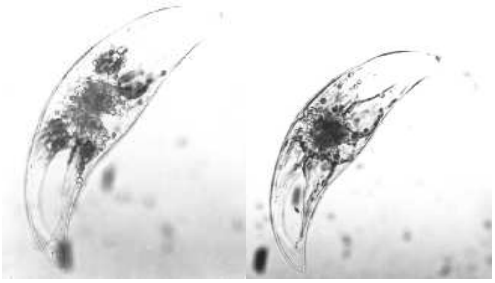

Figure 4.20: *The chloroplasts* of Pyrocystis lunula *change their position in a circadian way. During the night they contract into the cytoplasmic strips of the four horns (left), during the day they are expanded in the center. Photographs courtesy Hardeland*

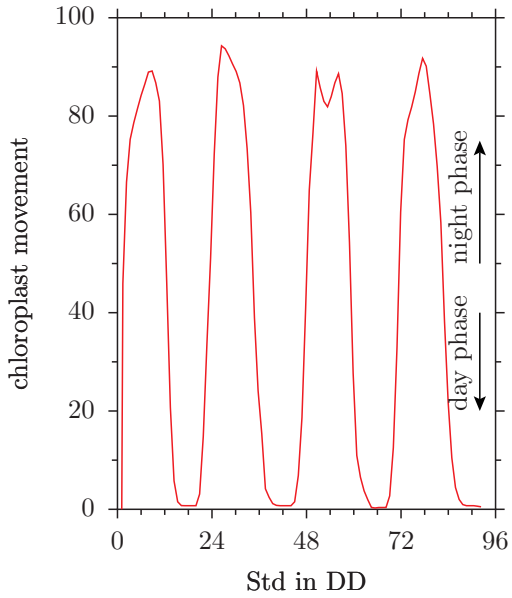

Figure 4.19: Pyrocystis noctiluca *exhibits a circadian movement of the chloroplasts in continuous darkness (percentage of cells, in which the chloroplasts have been expanded to less than a quarter of the cell surface). High values during night phase, low values during day phase. After [565]*

the day they protrude into the inner zone of the cell ([565] and figure 4.20, see also chapter 5). In continuous light the period is 21 to 23 hours. The rhythm disappears however in continuous light of intensities beyond 100 lux. The ultrastructure of the chloroplasts of *Pyrocystis lunula* fluctuates in a circadian way ([1366]). Plastid movement of *Pyrocystis acuta* is also under circadian control ([563]).

Circadian differences are also found in the ultrastructure of sub-cellular structures such as the thylakoid arrangement in the chloroplasts (figure 4.21). In the membranes of the theka the thylakoids lie closer together during the subjective night (CT 18) (upper part of figure) as compared to the situation during the subjective day (CT6, lower part of figure, [621]). At the subjective day the thylakoids possess two lamellae, at the subjective night three.

The photosynthetic unit in the thylakoid membrane shows differences during the course of a (circadian) day: In the (subjective) night a part is uncoupled from the electron transport. The association and dissociation of the antennas of the photo-

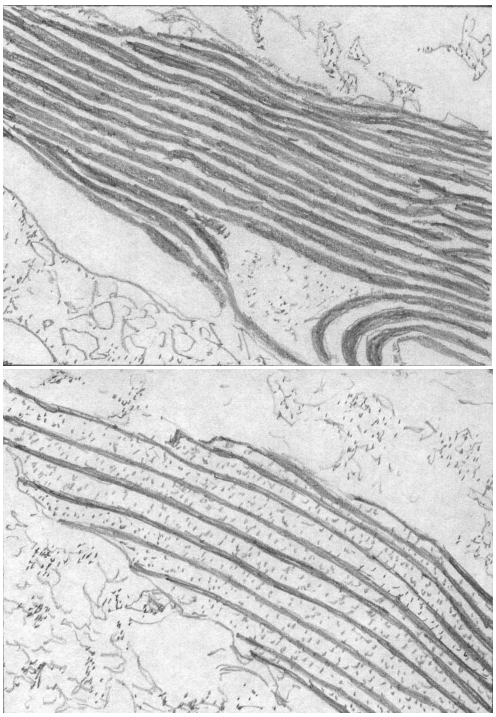

Figure 4.21: *The thylakoids in the chloroplasts of* Lingulodinium polyedrum *exhibit circadian differences. During the subjective night (CT 18) the thylakoids are closer together (upper part of figure) as compared to the subjective day (CT6, lower part of the figure). From [621]*

system II varies rhythmically. In this way the activating energy between photosystem I and photosystem II is distributed differently. [1304] studied the cause of the circadian oxygen production. They found in using the electron acceptor methylviologen, that the electron flow in the photosystem I is constant, whereas it fluctuates in a circadian way in photosystem II. Therefore only changes in photosystem II are responsible for the photosynthesis rhythm in *Lingulodinium* (figure 4.22, see also next section).

4.4 Circadian rhythms in photosynthesis

Structural changes in the photosynthetic apparatus pointed out already the influence of circadian rhythms on photosynthesis. A number of events in *Lingulodinium* are involved: CO_2-uptake ([578]), light reactions in the photosystem II[6], chlorophyll fluorescence and -breakdown[7] are examples. Photosynthesis is, however, not part of the clock: If it is inhibited with DCMU (blocks the electron flow in photosystem II), the clock continues to run ([1447]).

CO_2-fixing enzymes show no rhythm. The O_2 uptake is also constant. Respiration is therefore not responsible for the cir-

[6] the quantum yield of the photosynthesis and the electron flow through the photosystem II of *Lingulodinium* in weak continuous light fluctuates periodically. This is also the case if it is disconnected from the photosystem I. The uncoupled photosystem I however shows no circadian fluctuations in the electron flow.

[7] The breakdown of chlorophyll is lower during the day as compared to the night. Circadian ion fluxes through the thylakoid membrane are involved ([1441]). The antenna system of photosystem II and the organization of the antenna pigments seem to oscillate in a circadian way ([797]).

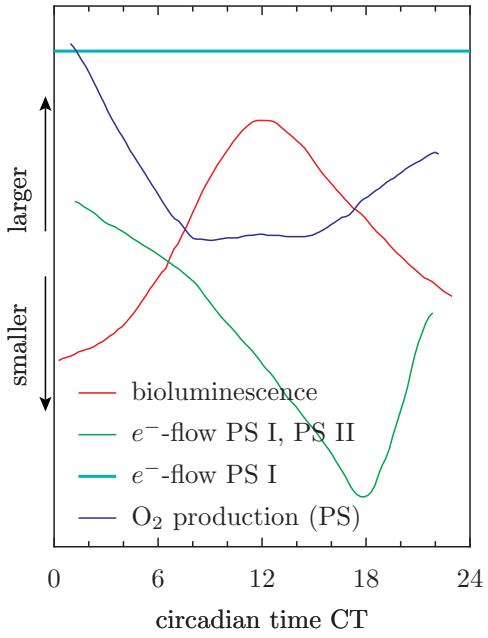

Figure 4.22: *Cause of circadian oxygen production in* Lingulodinium polyedrum. *Photosynthesis (green curve) fluctuates in a circadian manner (oxygen production was measured). It is low during the night phase, at which bioluminescence is high (red curve). Using the electron acceptor methylviologen it was shown that the electron flow in the photosystem I is constant (bluegreen straight line), whereas it fluctuates in a circadian way in photosystem II (blue curve). Therefore only changes in photosystem II are responsible for the photosynthesis rhythm in* Lingulodinium. *The x axis represents circadian time CT. No measures given at the y axis, but see [1304]*

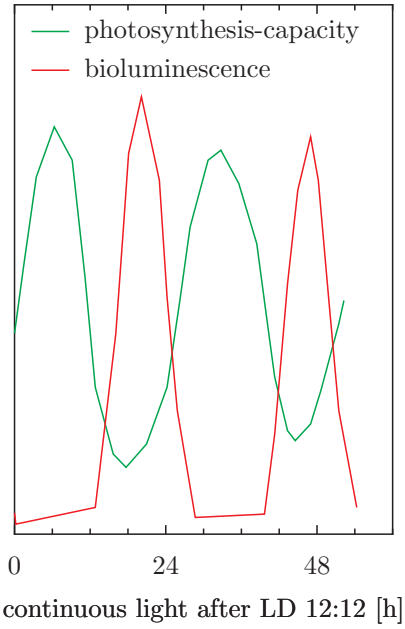

Figure 4.23: *Photosynthetic capacity in* Lingulodinium polyedrum *(green curve): Samples were supplied at different phases with $C^{14}O_2$ for 15 minutes in strong light. It was determined, how much C^{14} was taken up. Additionally the time course of bioluminescence (red curve). After [578]*

cadian rhythm. In the middle of the subjective light period the oxygen development is high, in the middle of the subjective dark period low (figure 4.23).

Since the density of a cell changes with photosynthesis, a circadian rhythm in a single cell could be demonstrated using a Cartesian diver (figure 4.24, [1437], http://de.wikipedia.org/wiki/CartesischerTaucher). The rhythm disappears at higher light intensities, as it does also in a population. The damping is therefore not based on a desynchronization of the rhythms between the single cells of a population.

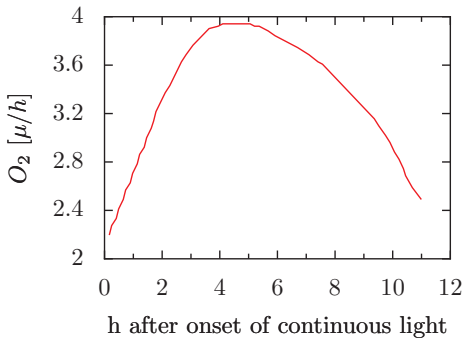

Figure 4.24: *The course of photosynthesis of a single* Lingulodinium *cell during the light period of a day was determined with a Cartesian diver. The density of the cell depends on the oxygen production. With it the position of the Cartesian diver (in which the cell is situated) changes in the container. After [1437]*

4.5 Cell division

Cell division is of paramount importance for organisms. To reproduce, the cells have to divide. The length of the cell cycle is determined mainly by the G_1 phase. The cycle might take anywhere between 8 hours to 100 days. Once the 'restriction point' is reached or induced, the cell cycle can not be stopt anymore. The cells go through the S-phase (DNA-synthesis, DNA-doubling), G_2 phase (preparation for mitosis) and M-phase (mitosis, that is division of the nucleus and cytokinesis) until the daughter cells are formed.

Cell division in a population *of Lingulodinium* cells kept in a light-dark cycle occurs mainly in the morning and under continuous light in the subjective morning (that is when light would normally begin). It is thus under circadian control. The generation time of an individual cell is under the light conditions used 6-7 days as an average (figure 4.25, [1442]).

The cell division cycle is not a circadian

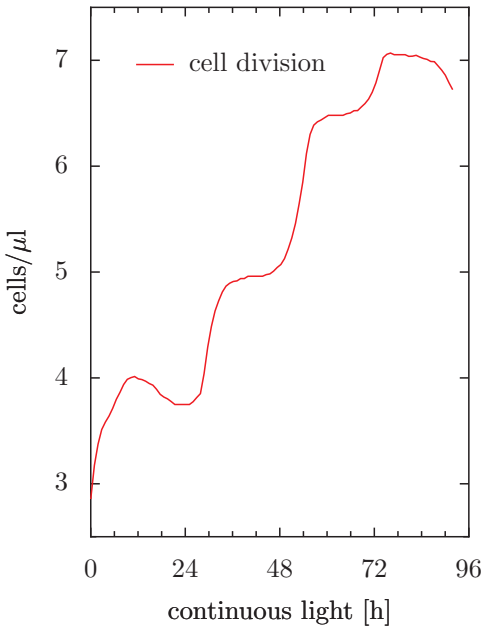

Figure 4.25: *Circadian rhythm of cell division in* Lingulodinium polyedrum*: The number of cells per µl increases in spite of continuous light (of 3000 lux) not gradually, but step-like. Circadian time CT shown on x-axis. Period length about 24 hours. After [1511]*

rhythm by its own, but is controlled by a circadian clock ([1443]). This control occurs via 'gating', meaning that the cell divisions occur in certain time gates ([1511]). Mitosis is generally found toward the end of the dark period or somewhat earlier, in *Gymnodinium splendens* however at the early evening. Cytokinesis takes one hour in *Lingulodinium*. In *Pyrocystis fusiformis*, a non-moving dinoflagellate with a 4-5 day cell cycle, a change in form occurs parallel to the cell cycle ([1449]). The cell division rhythm is not connected with the bioluminescence rhythm: cells, which do not divide, show still a circadian bioluminescence, and colchicine-treated cells likewise.

The cell cycle can be synchronized by sieving out cells according to their size ([653], [654]). After cell division the phase of the circadian rhythm is transferred to the daughter cells ([652]).

Under optimal conditions cell division is solely controlled by the mechanism of dividing. At suboptimal conditions an ultradian clock controls division (*[913]*). Under conditions which allow slow growth only the circadian clock takes over (gating, [910]). Different models have been proposed to simulate the cell cycle, from deterministic models to models with probabilities of transients, in which the cell volume changes this probability ([1495], review [910]).

4.6 Circadian rhythms of enzymes

Circadian rhythms were found in a number of enzymes in *Lingulodinium*. The nitrate-reductase is one of them (figure 4.26 and [1199], [462]). It is the first enzyme of the nitrogen assimilation pathway, converting nitrate into nitrite. Its concentration shows a circadian rhythm with a maximum at the (subjective) day phase. Another enzyme, superoxide dismutase, has its highest activity likewise at the day phase ((figure 4.26 and [240]). Whether its concentration changes in a circadian way is not known. This enzyme is a superoxide anion scavenger. In the case of RUBISCO, the most abundant enzyme in the biosphere, because it fixes CO_2 during photosynthesis, the activity changes in a circadian way while the amount of the enzyme stays constant ([955]). In contrast to these enzymes tyrosine aminotransferase exhibits its maximal activity at night ([532]). This was true also for the enzyme luciferase involved in the bioluminescence rhythm ([342], [706]). Generally protein synthesis during the day-phase is higher as compared to night phases. Most heat-shock-proteins are synthesized constantly, but some of them fluctuate in a circadian time course with a maximum around CT15. Ribosomal proteins are phosphorylated in a circadian way ([406]). If protein phosphorylation is inhibited with 6DMAP, the period is lengthened. At higher concentrations the rhythm disappears ([246]).

4.7 Effect of substances on the circadian rhythm, membranes

Different substances influence the circadian rhythm of *Lingulodinium*. As mentioned before, protein synthesis seems to be involved in the circadian mechanism of *Lingulodinium*. But membranes seem also to play an important role. This is interesting, because the fluidity of membranes is temperature-compensated. In

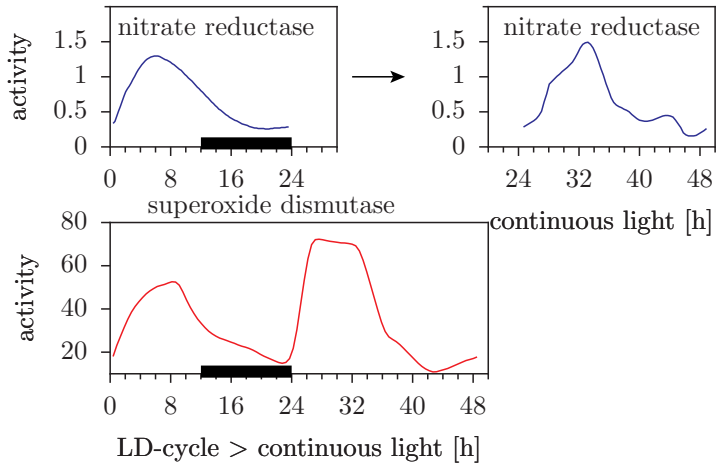

Figure 4.26: *Circadian rhythm of nitrate-reductase (top) and superoxide dismutase in* Lingulodinium polyedrum. *Top left: In a light-dark-cycle of 12:12 hours white light of 150 $\mu E/m^2 sec$. Right: In continuous light (hours after onset of continuous light 35 $\mu E/m^2 sec$). Activity of nitrate-reductase in relative units per mg. After [1199]. The superoxide dismutase (below) shows high activity during the light period. During the dark period the activity decreases and reaches a minimum at the end of the night. During the following continuous light this rhythm continuous, it is thus circadian. After [240]*

this way the temperature-compensation of the period length of circadian rhythms could be explained (see also 5.6). Membrane active substances such as K^+, Li^+, D_2O, valinomycin ([1440]), alcohols ([1446], [1458]), vanillic acid (depolarizes membranes, [769]) all influence circadian rhythms. This speaks also in favor of membranes being important for circadian rhythms. On the other hand metabolic differences could be responsible or the protein synthesis could be influenced. To test this, membrane properties were changed and measured using fluorescence polarization technique. In doing so, no correlation was found between membranes and period length ([1334]). The activity of membrane-bound enzymes and membrane potentials could, however, also change.

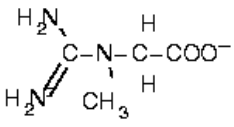

Figure 4.27: *Chemical formula of creatine. In [1256]*

Other substances such as acetaldehyde ([1456]) and catecholamine ([560]) influence also the rhythm, whereas inhibitors of respiration, of photosynthesis ([1441]), of organelle-ribosomes, cAMP and DNA-synthesis have no effect.

Creatine (figure 4.27), a storage form of ATP and a shuttle for energy rich phosphate between mitochondria and energy-consuming sites, shortens the period from

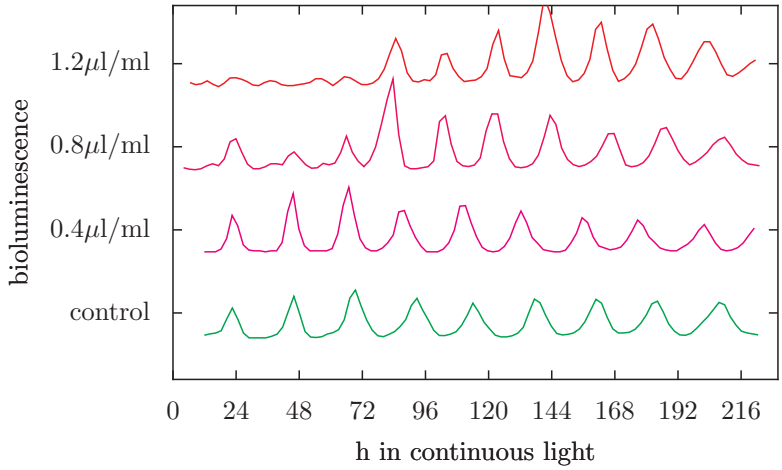

Figure 4.28: *Effect of 5 to 10 µM creatine on the period length of the bioluminescence-rhythm of* Lingulodinium polyedrum. *The algae were first kept in a 12:12 hour light-dark-cycle (light intensity 100 µE/m²sec) and afterward in continuous light (40 µE/m²sec). With increasing concentrations of creatine the period shortens. Time in continuous light plotted on the x-axis (in hours). After [1256]*

23 to 18 hours (figure 4.28, [1256], [1258]). It amplifies the phase shifting effect of blue light and the phototaxis. Creatine seems, however not to occur naturally in *Lingulodinium*. There exists, instead, another substance, Gonyaulin, which shortens the period[8]([1256], [1255]).

4.8 Rhythms in other dinoflagellates

There are a number of further dinoflagellates which have been shown to possess circadian rhythms. Some of them were mentioned before. It is certainly desirable and useful for comparative purposes to study their rhythms more detailed.

Bioluminescence and a rhythmic modulation of it was found also in *Ceratocorys horrida* ([863]). Its time course is similar to the one in *Lingulodinium polyedrum*, but the flash and the amount of light emitted is stronger. In *Pyrocystis lunula* ([1450]) a flash- and glow-rhythm has also been found, which is stronger as in *Lingulodinium polyedrum* and more light-sensitive. The glow rhythm is however not controlled by a circadian clock. Apparently the physiological control of the luciferase differs from that in *Lingulodinium polyedrum* ([241]). Hundred times more luciferine seems to be in this alga as compared to *Lingulodinium*. Furthermore there is no luciferin-binding protein in this alga.

In *Pyrocystis fusiformis* bioluminescence is circadian ([1449]). The rhythm is more pronounced as compared to *Lingulodinium*. As in *Lingulodinium*, the period length of *Pyrocystis* depends on the light quality (red, blue) and on the light intensity ([241]). The photosynthesis, cell division and movement of chloroplasts are controlled by a circadian clock ([1449]).

[8] Gonyaulin has a structure which is similar to a precursor of ethylene

5 Rhythms in algae: *Acetabularia*

Acetabularia *is another alga in which several circadian rhythms are observable. Due to its large size parts of it can be studied in respect to these rhythms and grafting experiments are feasible. They allow to find out the role of the nucleus for the circadian clock. Oxygen production, chloroplast migration and electrical potentials have been used as hands of the clock. A model of the circadian mechanisms was proposed and tested.*

Another alga which exhibits circadian rhythms and has been used for studies quite extensively because of its exceptional size is *Acetabularia*. It belongs to the *Dasycladaceae*, a very old family which existed already 500 million years ago. Depending on the species this unicellular alga has a size of a few millimeters up to 25 cm (the latter is *Acetabularia major* in the streets of Torres in Australia and in Papua-New-Guinea). The algae are tube-like with a root-like rhizoid. In the reproductive stage they form a hat (the algae are therefore called "umbrella" in Italian, "mermaids wine glass" in the United States, figure 5.1). Most *Acetabularia* are found in shallow waters of the coasts of tropical and sub-tropical seas.

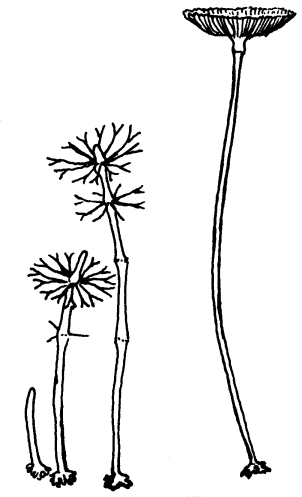

Figure 5.1: *Different stages of the unicellular alga* Acetabularia mediterranea. *The shortest is a germinating zygote (originated from two gametes), which are attached to the ground by a rhizoid. It extends to a stalk and forms whorls which disappear later. Finally a hut is formed, which produces in numerous chambers many cysts. In the cysts the gametes are formed. The alga reaches about 50 mm length. Found in the Mediterranean sea and the western Atlantic. After [494]*

5.1 Circadian phenomena

Acetabularia can be kept in the laboratory in artificial sea water and experiments can be performed with them. In this way daily and circadian rhythms of oxygen production during photosynthesis ([1463]), of enzyme activities ([610]), of chloroplast mi-

gration and of electrical potentials were found ([1352], [159], [815]).

Since the nucleus is seated in the foot (rhizoid) of the alga, grafting experiments could be designed to clarify the significance of the nucleus for the circadian rhythms ([1352]).

The rhythms can be synchronized by a light dark cycle. Light pulses and dark pulses shift the phase of the rhythm. Blue is the most effective light. It also reinitiated the circadian rhythm of the electrical potential and of chloroplast migration after the algae have been kept in continuous darkness ([132]). Furthermore the number, form and ultra-structure of the chloroplasts changes in a diurnal fashion (round during the dark period, oval during the light period, see [1505] and also section 20, page 449). The RNA-synthesis varies in a circadian manner ([1504]). The rhythm is temperature-compensated with a $Q_{10} < 1$ ([744], [91]). [1355] showed that occasionally the circadian chloroplast migration is shifted by an 8 hour dark period, whereas the rhythm of the electrical potential stays unshifted. This observation indicates the existence of two clocks.

5.2 Recording methods

Photosynthesis in *Acetabularia* was measured by using the oxygen production. A polarographic method was used ([1000]). A platinum-electrode is positioned in the sea water which had been pumped through a vial containing a single *Acetabularia* plant. The recorded voltage is a measure of the oxygen content (figure 5.2).

An automatic recording device was developed ([159], [1354]), which could measure 60 *Acetabularia* algae simultaneously. Scanning occurred every 20 minutes. The data were stored on a computer, graphically displayed and analyzed. The records show circadian changes due to a circadian rhythm in photosynthesis. The O_2 rhythm is observable also under continuous light. The electrical potential of the cells was recorded with a micro-electrode, amplified and plotted on a recorder or with an automatic long term recording unit on the monitor of a computer ([159]). It fluctuates in a circadian order.

The chloroplasts migrate during the night into the rhizoid at the base of the alga and during the day into the upper parts of the alga. Again this phenomenon is controlled in a circadian way. It is observable under the microscope and can be recorded automatically with light beams ([815], *[1354]*). The form of the chloroplasts changes also in a circadian fashion ([1501], [1502]).

5.3 Significance of the nucleus for the circadian rhythm

Because of its exceptionally large size *Acetabularia* can be used for a number of experiments which are difficult or impossible to do with other objects. For instance the role of the nucleus in the circadian mechanism can be studied. The cell is anucleated easily during its vegetative phase. Since the nucleus remains at that time in the rhizoid, this part has just to be cut off. The nucleus of another cell can be washed and implanted into such an anucleated cell fragment ([554]).

Using these methods the role of the nucleus for the circadian rhythm was studied by [1445], by [1351], by [744] and more closely by [1463]. The result was that the

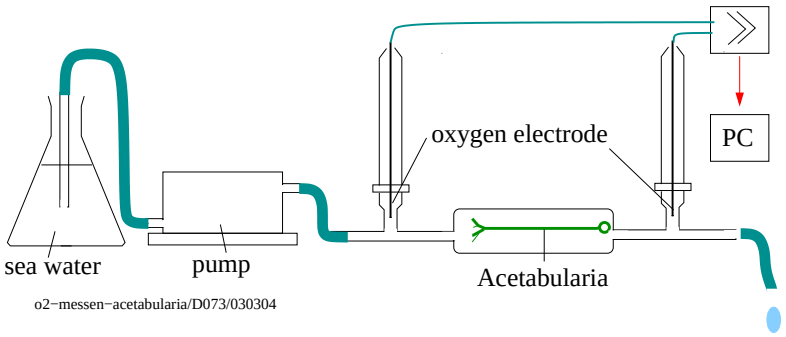

Figure 5.2: *The* Acetabularia *alga is positioned in a glass vial and continuously supplied with fresh sea water by a pump. The oxygen content of the water is measured before and behind the alga with oxygen electrodes. The difference of the recorded values is amplified and stored on a PC. It is a measure of the photosynthesis of the cell. After [1353]*

O_2-production during photosynthesis remains rhythmic in an anucleated cell (figure 5.3).

The oscillation is continuing in both the nucleus-containing and the nucleus-lacking part. This indicates that the oscillator is located in the cytoplasm. The integrity of the cell is not required for the circadian rhythm to occur. Even small cell fragments show still a circadian rhythm. Why is this so? It turned out that the mRNA of *Acetabularia* is stable for weeks, especially in the absence of the nucleus.

The nucleus plays, however a role: If rhizoids of *Acetabularia* are grafted on stalks of algae the phase of which was shifted, the rhythm in O_2-production in continuous light is determined by the rhizoid which contains the nucleus (figure 5.4, [1351]). To exclude cytoplasmic effects (as for instance due to mRNA), only the *nuclei* of algae which had been phase shifted where implanted. The rhythm was again determined by the nucleus. If the rhizoids and the upper parts of an *Acetabularia* were illuminated by different light dark cycles, the rhythm of O_2-production corresponds to the one under which the rhizoid was kept. [1502] grafted arrhythmic stalks of *Acetabularia* on rhizoids of rhythmic algae. Afterward a rhythmic O_2 production was observed.

Thus a paradox is seen: Although an anucleated *Acetabularia* cell exhibits a circadian rhythm, phase is according to these authors determined by the nucleus.

5.3.1 Translational membrane model of Schweiger

Since anucleated *Acetabularia* show a circadian photosynthesis, apparently a continuous transcription of the genome of the nucleus is not needed for the oscillations to occur. This is further corroborated by the finding, that an inhibitor of transcription in the nucleus, actinomycin, does not interfere with the rhythm of the algae ([1001]). Inhibitors of transcription in organelles such as rifampicin have also no influence on the circadian rhythm ([1001], [1503]). Translation is, however, important for the circadian rhythm, since inhibitors of translation such as cycloheximid prevent the circadian oscillation ([1001, 744]).

Based on these results Schweiger pro-

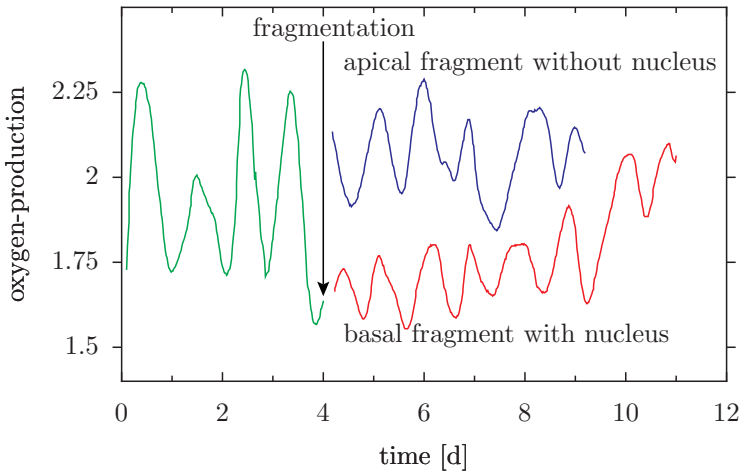

Figure 5.3: O_2 *rhythm before (vertical line) and after fragmentation of an* Acetabularia mediterranea *cell in an anucleated apical (upper curve behind vertical line) and a nucleated basal part (lower curve behind the vertical line). After [1353]*

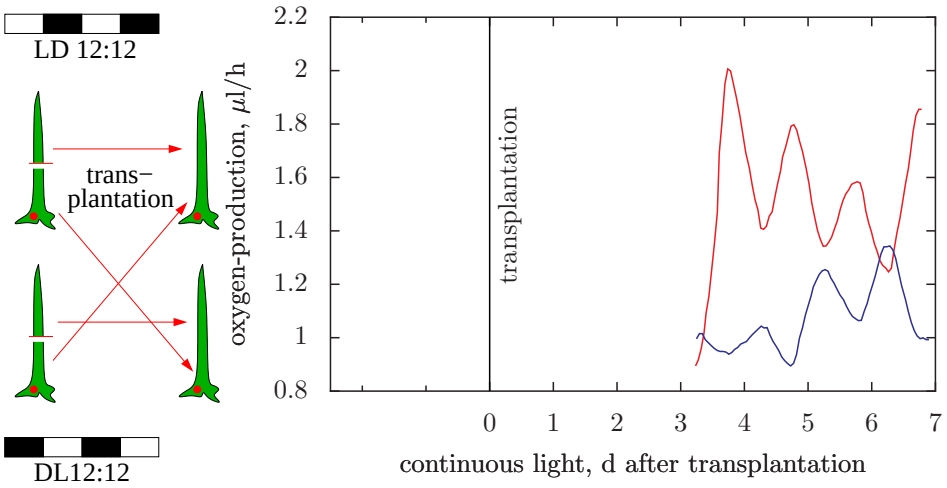

Figure 5.4: *Two* Acetabularia *algae were kept in an inverse light-dark-cycle (see LD cycle 12:12 and black and white bars before transplantation above (a) and below (b)). The rhizoid with the nucleus (marked red in the figure) of (a) was grafted to the stalk of (b). Onto the rhizoid of (b) the stalk (marked red in the figure) of (a) was grafted. Recordings started after three days and show inverted rhythms. A comparison with controls (not illustrated) show, that the nucleus determines the phase of the product of the grafting. After [1351]*

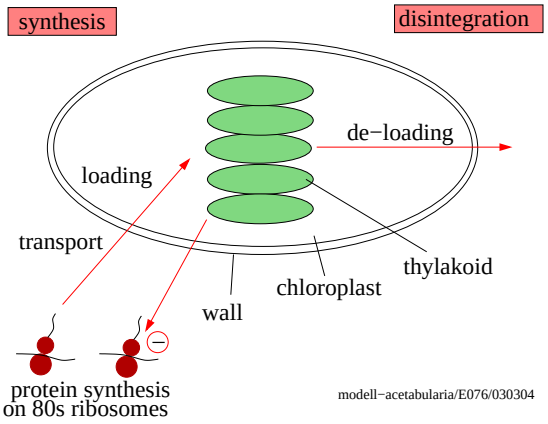

Figure 5.5: *Translational membrane model of the circadian mechanism of* Acetabularia *according to Schweiger and Schweiger. Essential proteins are made by 80s ribosomes and transported via the cytosol into the chloroplasts. There they are incorporated into the thylakoid membranes. After loading, the protein synthesis is inhibited, the essential proteins are metabolized, until the thylakoids are discharged. Now the assembly can start again (left part of figure). After [390]*

posed a translational membrane model (figure 5.5). Central components of the oscillator are essential membrane proteins in the thylakoids of the chloroplast. They influence the permeability for ions. Feedback inhibits the translation of the membrane proteins on 80s ribosomes. The membrane proteins are slowly broken down and the permeability of the membranes for ions changes. The inhibition of translation stops.

What are the experimental supports for this model and how does it explain certain properties of circadian clocks such as temperature compensation? [572] found a nucleus-coded protein P230 in the chloroplast fraction of nucleated and anucleated *Acetabularia*. It is synthesized in a circadian way under constant conditions. Cycloheximid, an inhibitor of protein synthesis on 80s ribosomes, inhibits its translation in a phase-dependent way. It furthermore shifts the circadian rhythm of photosynthesis as a function of the phase. P230 could thus be one of the essential proteins in the model.

The temperature compensation of the circadian rhythm in this model is envisioned in the following way (see figure 5.6): The translation of the essential membrane proteins at the 80s ribosomes has a Q_{10} of 2-3, but the integration of the proteins into the membranes of the chloroplasts has a Q_{10} of less than 1 (because of the lower state of order at higher temperatures the integration is more tougher).

[1583] has repeated the experiments on nucleated and anucleated *Acetabular*ia. He recorded, however, instead of the O_2-evolution the circadian chloroplast movement in the rhizoid by using light beams in a way described by [1330] (figure 5.7). Instead of two recorded data per day as in the case of [1351] he obtained every minute a record and averaged them over an hour. Algae with a rhizoid showed a period length of 25.4 hours under constant conditions, algae without a rhizoid 26.2 hours. Thus the nucleus does affect the rhythm, although to a small extent. Too small, to be detected by the low sampling rate used by Schweiger. Controls showed phase differences up to 4 hours between each other. Woolum could not reproduce the results of Schweiger by illuminating the upper part of the algae and the rhizoid. The nucleus did not exert a phase information on the upper part of the algae. Apparently the oscillator needs a stable mRNA, but does not need mRNA synthesis. Actinomycin, which inhibits mRNA synthesis, has no effect on the phase. Only the amplitude of the rhythm was reduced ([1448],

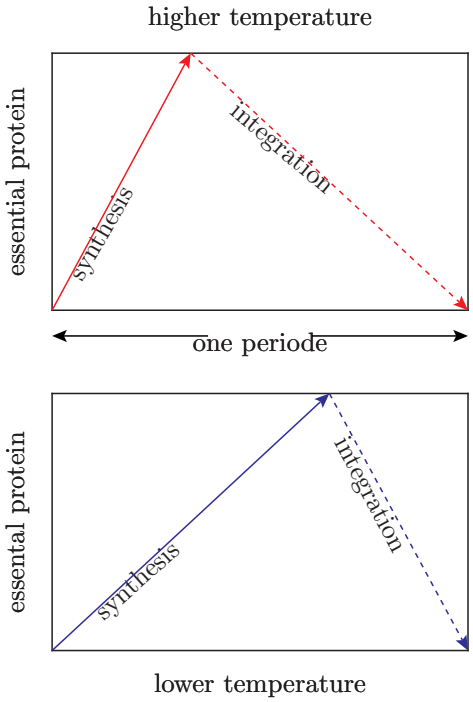

Figure 5.6: *Temperature compensation of the circadian rhythm of* Acetabularia. *At higher temperature synthesis of essential proteins occurs faster, but the integration into the thylakoids more slowly. At lower temperature synthesis is slowed down, but integration speeded up. In this way the length of the period depends only slightly on temperature. After [390]*

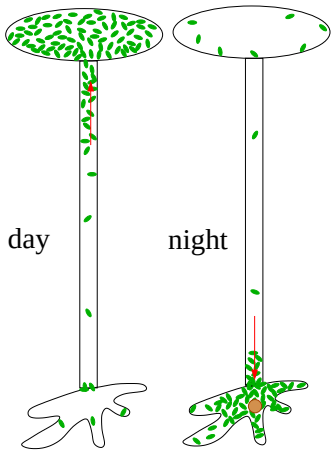

Figure 5.7: *Circadian chloroplast migration in an* Acetabularia mediterranea *cell. Chloroplasts accumulate during the day in the hut and the upper stalk (left), during the night in the rhizoid and the lower part of the stalk. After [1353]*

[1503]).

5.4 Several oscillators?

Are the different circadian oscillations in *Acetabularia* controlled by one clock? The chloroplast movement, electrical potential and oxygen production were recorded in one individual cell at the same time ([1354]). They stayed always in phase to each other, even if the temperature of the water was changed. This indicates, that either the different rhythms are driven by one clock, or there are different oscillators, which are strongly coupled to each other. An observation of [572] speaks in favor of the latter: The circadian chloroplast migration and the circadian change of the electrical potential are normally phase shifted (as a function of phase) by an 8 hour dark pulse during continuous light by the same amount. However, sometimes it happens,

that only one rhythm is shifted, not the other. This would indicate that two different clocks control the two recorded events.

5.5 Do cells interact with each other?

It was tested whether *Acetabularia*-cells interact with each other ([999]). 50 cells where kept in a 12:12 hours light dark cycle and than transferred into a vial containing a single *Acetabularia* cell which had been kept in a light dark cycle 12 hours out of phase (i.e. it had light, when the 50 cells were in the dark). Under constant conditions these cells did not influence the single cell. They kept their rhythm for the seven days of recording. *Euglena* and *Lingulodinium* do not influence each other either.

6 *Cyanobacteria* - Daily rhythms in prokaryotes

Only recently circadian rhythms have been found to exist in prokaryotes. They are expressed in photosynthesis, nitrogen fixation, carbohydrate synthesis, cell division. A luciferase expressing reporter gene was inserted allowing easy recognition of the circadian clock. Many clock mutants were obtained in which the circadian rhythm was changed or missing. Genes were identified which take part in the clock work. This allowed to study the clock mechanism and a model has been put forward. Clock mutants were also used to test for an adaptive significance of circadian rhythms.

If we want to study and finally understand the mechanism of circadian control systems, it is of advantage to use a rather simple system. It should of course possess a handy hand of the circadian clockwork, but not much in addition, so to speak a 'minimal system'. This is generally a good practice for studies of processes going on in organisms. If we would, for instance be interested in some aspect of glycolysis, yeast cells would present a nice minimal system. It has indeed been used profoundly for such studies. A minimal system should possess a simple structure and physiology. Rearing and keeping it alive should pose no difficulties. It should preferentially also be accessible to genetic and molecular biological methods. And finally the hands of the clock should be recordable in an easy way.

Human blood cells were claimed to be such a minimal system (figure 6.1). They are specialized in transporting the oxygen from the lungs to the tissues of the body. As all mammalian erythrocytes they lack in their mature and functional stage nuclei and possess no nucleic acids. They lack furthermore most other organelles usually found in cells such as mitochondria, and are therefore not able to perform respiration. They are tuned to their main task: bind oxygen to the hemoglobin molecule and deliver it all over the body. Besides, erythrocytes can be kept in vitro and are thus manageable easily in the laboratory. There is no difficulty in keeping them under constant conditions at the same temperature and at other conditions. And, last but not least, it was claimed that different enzymes fluctuate in a circadian way ([37], [36], [571]). This team observed activity changes in glucose-6-phosphate-dehydrogenase, acid phosphatase and acetyl cholinesterase. However, different attempts to repeat these important findings were not successful so far ([250], [251], [941], [1103]).

Another minimal system with circadian changes are seeds of bean plants. The dry seeds show an extremely low respiration rate, no nucleic acid metabolism and no synthesis. In spite of it the respiration was described to change in a circadian way ([176]).

What is common to the two systems is the complete lack of nucleic acid metabolism. This is an important is-

Figure 6.1: *Human erythrocytes. Cell in foreground with rough surface is white blood cell. After [758]*

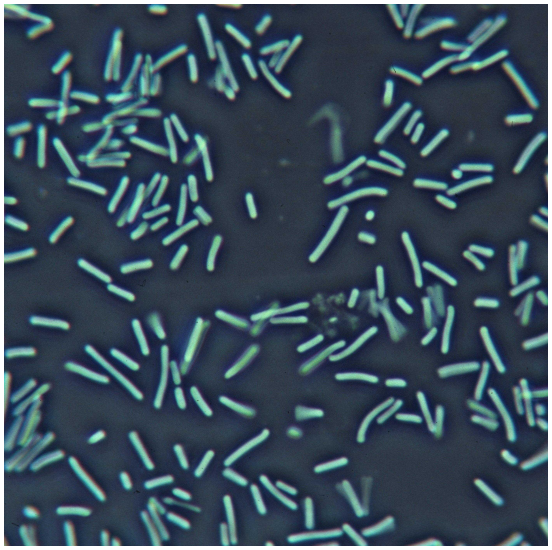

Figure 6.4: *Cells of* Synechococcus elongatus PCC 7942*, a Cyanophycea. Image kindly supplied by Takea Kondo, Nagoya University Japan*

sue, since several of the recently proposed models of circadian systems use feedback systems in transcriptional and translational events (see chapter 6, subsubsection 14.2.5, and section 16.8). It would therefore be of much interest to repeat these experiments carefully and to try to avoid any pitfalls.

A recently found and exploited minimal system are certain *Cyanobacteria*. For a long time it was assumed that circadian rhythms are found only in organisms with a true cell nucleus, so called eukaryotes. Prokaryotes were assumed to be without circadian clocks.

It came therefore as a surprise, when in the eighties of the last century several groups showed circadian rhythms to exist in *Cyanobacteria*. It was first discovered in an *Oscillatoria* (figure 6.5 and [1408]) and intensively studied in *Synechococcus*- ([530][1018], figure 6.4) and *Synechocystis* species. That photosynthesis of the *Cyanobacteria* can occur in the light only is obvious. But even under constant temperature and in continuous light *Synechococcus* produces during photosynthesis different amounts of oxygen. And it turned out that a circadian rhythm with a period length of 20 hours is responsible for it ([1018]). Nitrogen fixation, carbohydrate synthesis and photosynthesis, but also cell division and other processes are under circadian control (figure 6.2 and 6.3).

The finding of circadian rhythms in *Cyanobacteria* indicates that they probably evolved much earlier as anticipated so far. *Cyanobacteria* were found already in the flint stratum in Canada as fossils. They are therefore more than 3.5 billion years old. It is not known, whether they possessed already circadian rhythms, but they were able to fix nitrogen from the air (as shown by heterocysts in the fossils, specialized cells for this purpose in *Cyanobacteria*). The next record in the earths history for daily rhythms are much later, namely 420 million years ago. They were established in the epithek of corals and in the shells of nautiloids (see chapter 17).

Cyanobacteria are especially well suited for circadian studies. The control mechanism of transcription is better known in prokaryotes as it is in eukaryotes. Their

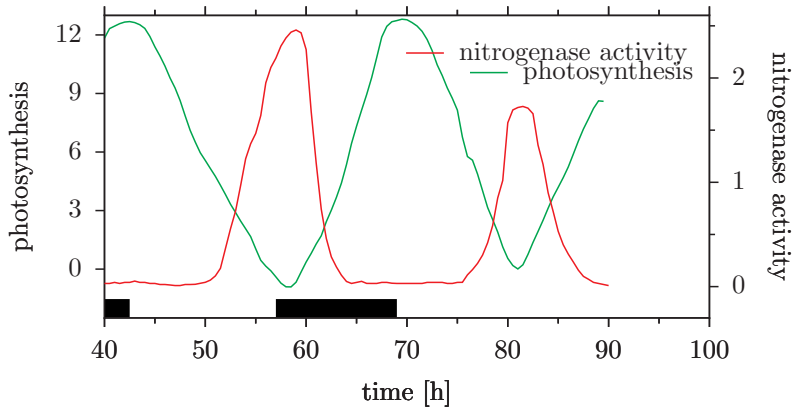

Figure 6.2: *Oxygen-formation during photosynthesis (green curve) and nitrogen-fixation by nitrogenase (red curve) in* Synechococcus *under 12:12h light-dark (left) and under continuous light (right). Nitrogenase-activity is high, if photosynthesis is low. After [1017]*

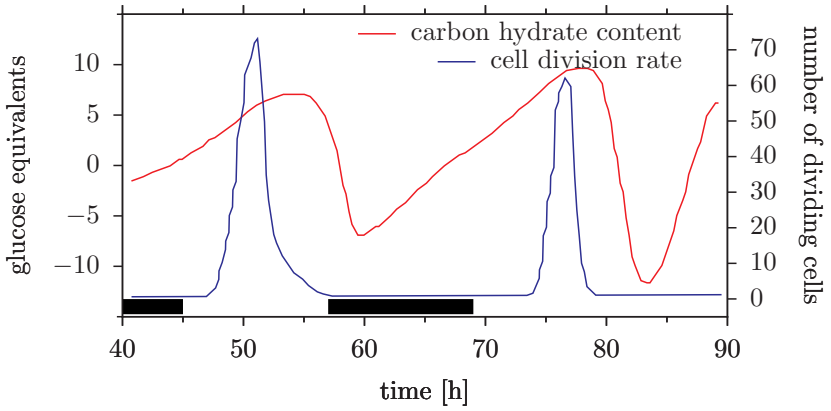

Figure 6.3: *Carbonhydrate synthesis (glucose-equivalents per ml and hour, red curve) and cell division rate (number of dividing cells, blue curve) of* Synechococcus *are circadian. After [1017]*

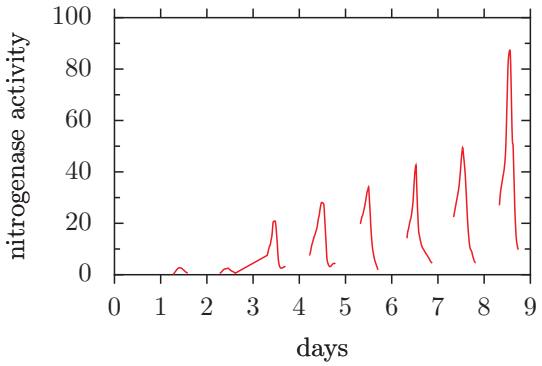

Figure 6.5: *The nitrogenase-activity of an* Oscillatoria spec. *(strain 23) was recorded in continuous light for eight days using acetylene-reduction. A circadian rhythm is shown. After [1408]*

genome is smaller and therefore saturating mutagenesis of clock genes can be performed. Molecular biological manipulations of *Cyanobacteria* are widely and extensively used. Furthermore the genome of the first *Cyanobacteria* have been sequenced completely ([742]) and more will certainly follow soon (for general informations on porkaryotes see [882]).

We will look at the main differences between prokaryotes and eukaryotes. Afterward, some circadian controlled events in *Cyanobacteria* are presented. More recent molecular genetic studies allow glimpses into the underlying mechanisms ([806], [702], [503], [807]). An artificial hand of the circadian clock was put into a *Synechococcus-* and *Synechocystis* species. It allows to monitor the course of the circadian clock in many populations simultaneously. Furthermore numerous mutations affecting the circadian system could be produced.

6.1 Differences between pro- and eukaryotes

In table 6.1 the most important differences between pro- and eukaryotes are compiled. Besides the size these differences show up mainly in the following: In eukaryotes transcription and protein synthesis occur in different compartments divided by a nuclear membrane, whereas prokaryotes do not possess a nucleus. A cytoskeleton of the eukaryotes allows cytoplasmic streaming to take place and thus intracellular transport. In prokaryotes without these compartments the structure would be destroyed, if the cytoplasm would stream. The cytoplasm of prokaryotes contains DNA, RNA, proteins and small molecules, but has no inner structures. By multiple replicons the control of cell division in eukaryotes differs from that in prokaryotes. Eukaryotes possess exocytosis, a Golgi apparatus, endoplasmic reticulum, cell compartmentation with mitochondria, plastids and other organelles. They contain non coding DNA, repetitive DNA, and more splicing of RNA which occurs more often. The genome of prokaryotes is lesser, the cells smaller, the mode of division differs. There is only one single and circular chromosome. Cell division can occur under optimal conditions every 20 minutes (that is, in 11 hours 5 billion cells would have formed, a number which corresponds to the number of people on earth). In this way prokaryotes can adapt faster to changing environmental conditions. Bacteria are from all cells the most widely distributed one on earth.

Cyanobacteria possess a photosynthesis apparatus, which corresponds to the one of eukaryotes. Due to additional pigments phycobilicyanin and phycobiliery-

Table 6.1: *The most important differences between pro- and eukaryotes*

	prokaryotes	eukaryotes
organisms	bacteria and cyanobacteria	unicellulars, fungi, plants, animals
cell size	1-10 μm length	5-500 μm length
metabolism	anaerobe, aerobe	aerobe
organells	none	nucleus, mitochondria, chloroplasts, endoplasmic reticulum and others.
DNA	circular in cytoplasm	very long, linear, many non coding regions, nuclear membrane
RNA and proteins	in same compartment synthesized	RNA in nucleus synthesized and processed, proteins in cytoplasm
cytoplasm	without cytoskeleton, without endo- and exocytosis	cytoskeleton made of protein filaments, cytoplasmic streaming, exocytosis
cell division	chromosomes depart by adhering to plasma membrane	chromosomes depart by spindle apparatus
cell organization	mainly unicellular	mainly multicellular, differentiation into many cell types

thrin they can, however, use wavelengths of light for photosynthesis, which eukaryotes are unable to absorb.

Prokaryotes offer thus a number of advantages for studying circadian rhythms: The control of transcription and translation is simpler, the genome smaller, the structure of the cells and the metabolism more fundamental.

6.2 Circadian rhythms

Let's have a look at an example of the diurnal rhythm of mobility found in different prokaryotes. Water samples were taken during a summer day at 6 o'clock in the morning from the surface of the Mendota lake (Madison, Wisconsin, USA) and from different depths up to 8 meters below the surface. The sampling was repeated every 3 hours until 21 o'clock. Under the microscope the number of *Aphanizomenon flos aquae*, a cyanobacterium, was determined for each sample by measuring the chlorophyll absorbance, and the profiles plotted for the different sampling times. The results are shown in the left curve of figure 6.6. Highest density shifts during the course of a day, and during the night there are only few cells found at the surface. Most cells sank to lower horizons of the lake which is reflected in the shift of the peak. The curve at the bottom of the figure shows the relative change in vertical distribution at the surface. It exhibits a daily variation.

Quite a number of mechanisms are controlled in a daily way in *Cyanobacteria*. In different prokaryots diurnal motility rhythms exist (figure 6.6). As many other planktonic *Cyanobacteria* cells *Anabaena flos aquae* contains gas vacuoles. They cause a change in buoyancy leading to algal blooms during the summer and fall. Diurnal vertical migration is also observed in *Oscillatoria*-populations ([451]).

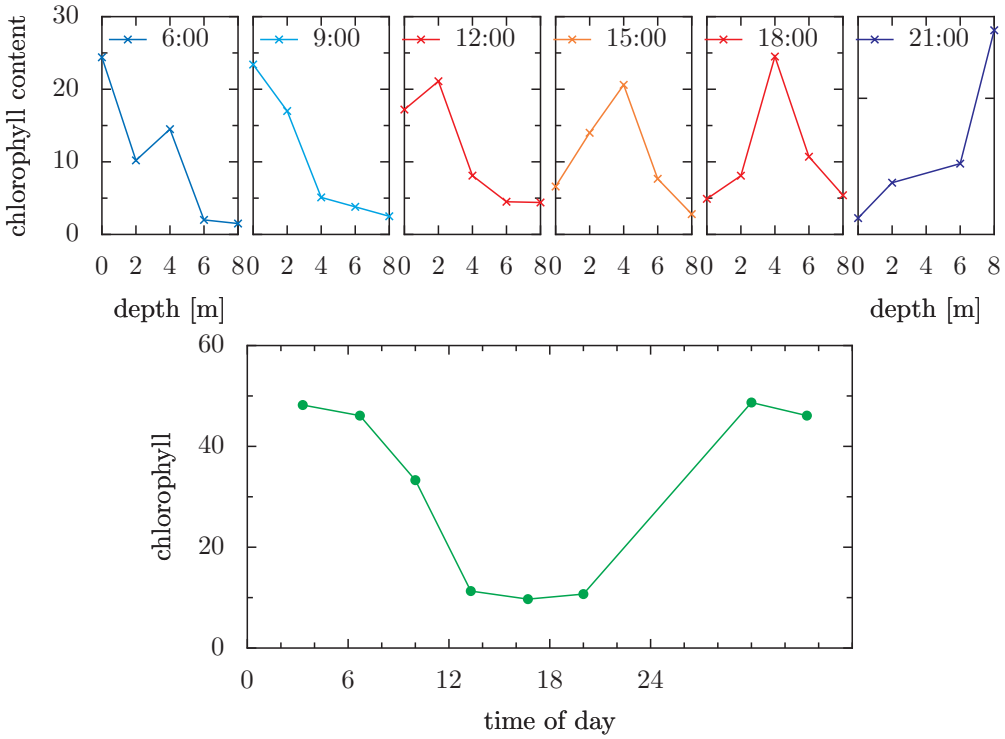

Figure 6.6: *Examples for the mobility rhythm of* Aphanizomenon flos aquae*, a* Cyanobacterium *in the lake Mendota during a summer day. Top: Density of the Cyanobacteria (amount of chlorophyll as a measure, y-axis) at different times of the day and in verious depths (x-axis). Bottom: Chlorophyll content (y-axis) varies with time of day. After [811]*

The oxygen production of photosynthesizing *Synechococcus* is also rhythmic. This is to be expected in a light-dark cycle, but is found also under continuous light conditions proving its circadian nature (figure 6.2).

Numerous *Cyanobacteria* are able to fixate nitrogen of the air. They play an important role in the nitrogen cycle between water/soil, plants, animals, and the atmosphere (see figure 6.7). The responsible enzyme nitrogenase is, however, inhibited by oxygen. Therefore a circadian clock in *Synechococcus* takes care, that this enzyme is active at times where no oxygen is formed, which is during the dark period of the day. Other hands of this circadian clock of the *Cyanobacteria* are carbohydrate synthesis and cell division ([1017] and figure 6.3).

Many *Cyanobacteria* can divide under optimal conditions faster than 24 hours. This is true also for *Synechococcus*. In spite of it other rhythms continue to be circadian (for instance [808], [1033], figure 6.8). The two events are, however, not independent from each other: Cell division is restricted to a time window and is 'forbidden' during the remaining time.

In *Synechococcus* this forbidden zone takes only a small part of the circadian cycle, whereas in eukaryotic cells such as *Euglena* it covers much of the circadian cycle and division of a mother cell might occur only once per 24 hours (figure 6.9). Apparently *Synechococcus* cells use different circuits for cell division and for circadian control of events. The circadian clock is completely independent of the cell division cycle, but cell division is influenced by a gating control of the circadian clock.

Circadian rhythms are not only found in *Synechococcus*. Photosynthesis, carbohydrate formation and nitrogen fixation

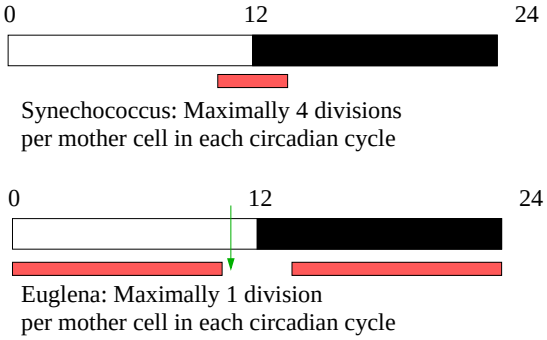

Figure 6.9: *Top: Cell division in* Synechococcus *PCC7942 is forbidden in a certain part (red rectangle) of the circadian cycle, but allowed outside of it, where under favorable conditions a mother cell might divide up to four times each cycle. Bottom: In contrast, in the eukaryotic alga* Euglena *the forbidden zone covers most of the circadian cycle and during the allowed portion a mother cell can divide only ones (green arrow). After [1033]*

show also a circadian rhythm in *Cyanothece* ([1333]). Likewise a filamentous *Cyanobacterium*, *Trichodesmium*, fixates nitrogen in a diurnal and a circadian way ([233]).

Another hand of the circadian clock is easily recorded by using a pH meter, but the detection of the rhythm is somewhat tricky and needs special mathematical treatment: The medium is acidified by *Synechococcus* in a circadian way ([777], figure 6.10). This occurs step-wise, where the steps are by about 24 hours apart from each other. The acidification could be due to the activities of proton pumps or to other proton transport mechanisms.

Transport processes are also influenced by the circadian clock in the uptake of different amino acids by *Synechococcus*. The uptake rates fluctuate in a circadian pattern ([231]).

All these different oscillations possess already the typical properties of circadian

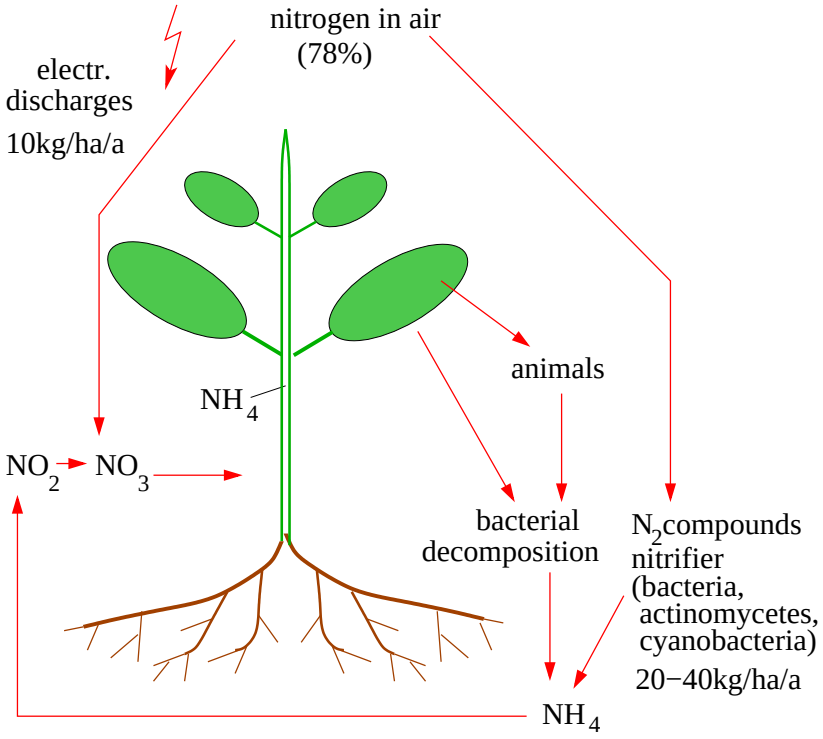

Figure 6.7: *Nitrogen cycle between air, plants, animals and decomposing organisms. After decomposition and bacterial nitrification NO_3 can be taken up by plants for amino acids, proteins, nucleic acids and other compounds. Electrical discharges of thunderstorms convert N_2 to NO_3, amounting to 10 kg per hectare and year. Nitrificants such as* Bacteria, Actinomycetes, *and* Cyanophycea *are able to fixate 20 to 40 kg N_2 per hectare and year.* Cyanophycea *in Indian paddy fields fixate 50 kg N_2 per hectare and year. After [281]*

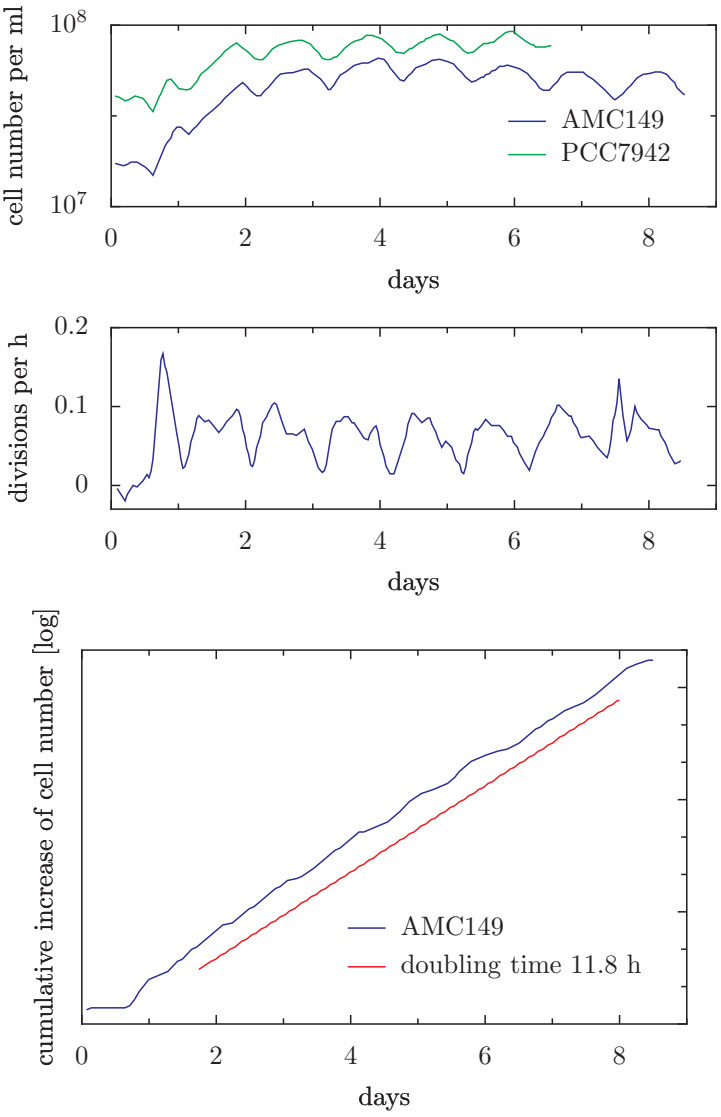

Figure 6.8: *Cell division in* Synechococcus *PCC7942 (green curve top diagram, 24.0 hr period length) and AMC149 (blue, 25.2 hr period). Last dark period of 12:12 LD cycles before recording ended at 0. Diagram in center: Blue curve data of top diagram replotted as instantaneous rate of increase in cell number compensated for rate of medium dilution (moving average of data). Lower diagram: Blue curve in top diagram replotted as cumulative increase in cell number ('logistic growth curve', calculated from rate of dilution and cell number). Red curve: doubling time of cell division. The diagrams show, that in spite of a doubling time of 11.8 hours the* circadian *rhythm continues. After [1033]*

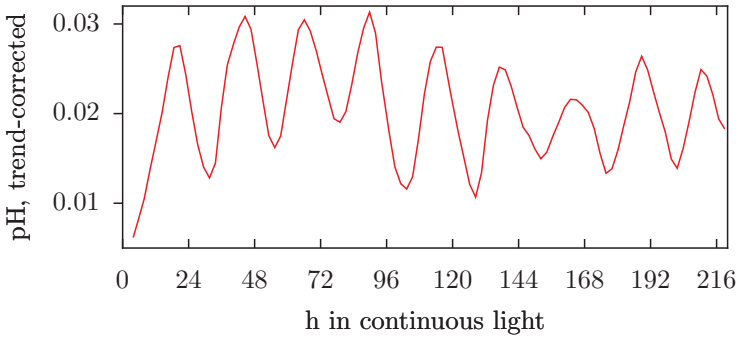

Figure 6.10: *The pH of the medium is modulated in a circadian manner by* Synechococcus. *The pH values are trend-corrected and the differences plotted. Continuous light after 12:12 hours light-dark-changes. After Kippert, unpublished.*

rhythms of eukaryotes: The period free runs under constant conditions, they are synchronizable by Zeitgeber and they are temperature compensated.

6.3 Luciferase-expressing *Synechococcus*

Provided a good screening method could be found, *Cyanobacteria* such as *Synechococcus* or *Synechocystis* would be almost ideal organisms to be used for mass screening for mutants in the circadian system. However, the different hands of the circadian clock of *Synechococcus* as described before where unsuited. Therefore an elegant trick was applied which was used also in other organisms to unravel the circadian clock: A reporter gene was inserted behind a promoter which is under control of the circadian clock. As a reporter the luxAB gene for bacterial luciferase was used. In this way *Cyanobacteria* were obtained which show a circadian luminescence ([809]).

The wild type-*Synechococcus elongatus* PCC 7942 was used, since it is well studied in molecular genetic terms. The *Synechococcus*-genome has $2.6 * 10^6$ base pairs and is smaller as that of *Escherichia coli*. The promoter P psbAI regulates normally the gene psbAI, which codes for the protein D1. D1 forms a dimer with D2, which binds to QB. It is an essential molecule participating in the electron transport in photosystem II (figure 6.11).

The luciferase-structural gene luxAB was obtained from the luminescent bacterium *Vibrio harveyi*. It was inserted into a reporter-plasmid and adhered to the promoter P psb AI in a 'neutral site' by homologous recombination (figure 6.12).

As a substrate for luciferase n-dekanal is used, an aldehyde. As expected a rhythmic luminescence of the cultures was observed. In non-transformed *Synechococcus*-cells mRNA was extracted at different phases and shown, that psbAI is indeed expressed under constant continuous light-conditions in a circadian rhythm (figure 6.13). Therefore the luminescence rhythm is brought about by a circadian control of the psbAI promoter ([809].

Two cultures being phase shifted against each other keep this phase relationship of the luminescence rhythm also under conditions of constant light

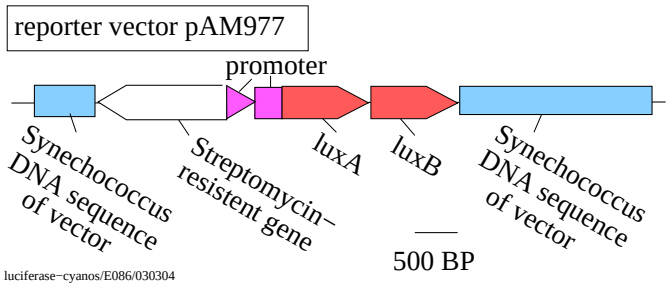

Figure 6.12: *Reporter vector pAM977 as a construct of the luxA/luxB luciferase-structural gene attached to the promoter PpsbAI of the photosynthesis apparatus of* Synechococcus. *After [809]*

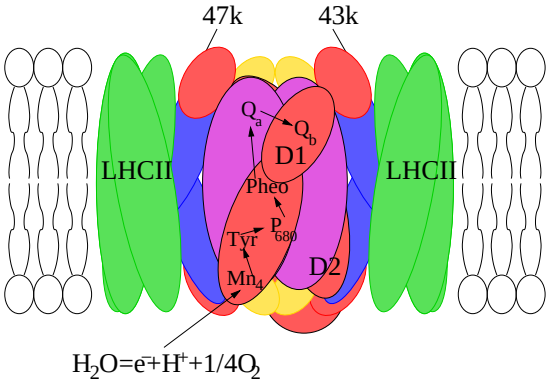

Figure 6.11: *Electron transport in the photosynthesis-system of* Cyanobacteria. *Electrons (e^-) are transferred after water hydrolysis through a chain to Q_b. D_1 and D_2 form a dimer which binds to Q_b. The gene psb AI codes for the protein D_1 and is under the control of the promoter P psb AI. The promoter is under the influence of the circadian clock. After [809]*

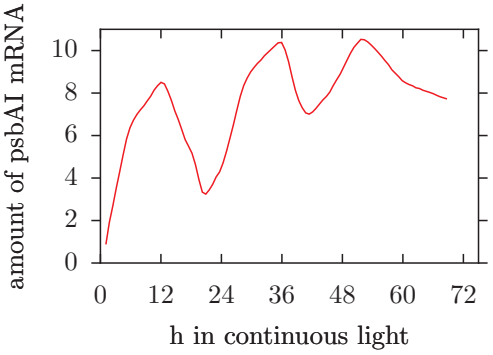

Figure 6.13: *At different phases mRNA of psbAI was extracted from* Synechococcus *and the amount determined. The mRNA is expressed in a circadian way under conditions of continuous light (following 12:12 hour light-dark-changes. After [809]*

(figure 6.14). This shows that the rhythm is indeed endogenous and not caused by an external Zeitgeber (which might have been present in spite of the continuous light). The rhythm occurred also under continuous darkness ([20]).

Using dark pulses the circadian rhythm under continuous light conditions can be phase shifted (figure 6.16).

In the meantime a luciferase gene has been introduced also in a *Synechocystis*-strain PCC6803 and a circadian rhythm of luminescence was demonstrated.

The rhythm persists also in continuous darkness in a circadian mode. Normally the rhythm would damp out quickly in darkness, but if glucose is added to the substrate it continues for at least 7 days ([20]). With light pulses the rhythm of cultures kept in continuous darkness is phase shifted (figure 6.15, [20]). Using suitable mutants this opens up the possibility to track the path of the light from its perception and the signals which finally influence the phase and amplitude of the clock.

The genome of *Synechocystis* has been sequenced in the meantime completely ([20]). It possesses different molecules, which could serve as transducers of light: photoreceptors (for instance a phytochrome), a two-component-system, which transfers a signal and adenylat cyclase. If certain genes are switched off or are over-expressed, one could find out whether the molecules coded by these genes participate in the transduction pathway of the light.

At different environmental temperatures the period length of the circadian oscillator of *Synechococcus* stays almost constant ([1436], *[809]*). Thus the circadian clock of this *Cyanobacterium* is also temperature-compensated (figure 6.17). The Q_{10} values is 1.1 and thus in a range, which is characteristic for circadian rhythms of eukaryots.

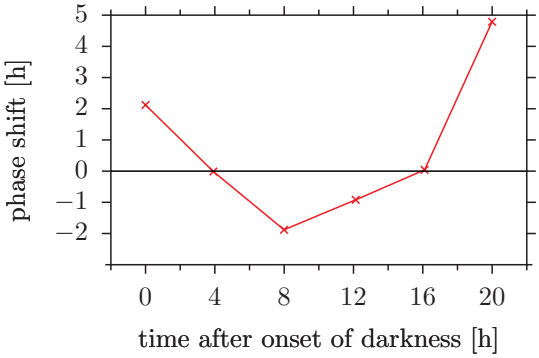

Figure 6.15: *Light pulses shift the circadian rhythm of bioluminescence of* Synechocystis *under continuous darkness differently depending on the time of application. After [20]*

6.4 Finding clock mutants

To find clock-mutants in *Cyanobacteria* two things are necessary: Mutations have to be induced which is usually done by treating the cultures with a mutagenic substance and rearing colonies from many treated individuals on agar plates. Secondly, the circadian rhythm has to be monitored. For this purpose bioluminescence was recorded with a sensitive video camera every 30 minutes and evaluated separately for the individual colonies ([806]). Most colonies showed no change in their clock properties. But some showed differing period lengths or amplitudes of their circadian luminescence rhythm (figure 6.18).

The interpretation of the result of such a mutation is not always easy. To give an example: A mutant was found with a period length of 22 hours only, whereas the period of the wild type was 24 hours. It was called sp22. This mutant can, however, not

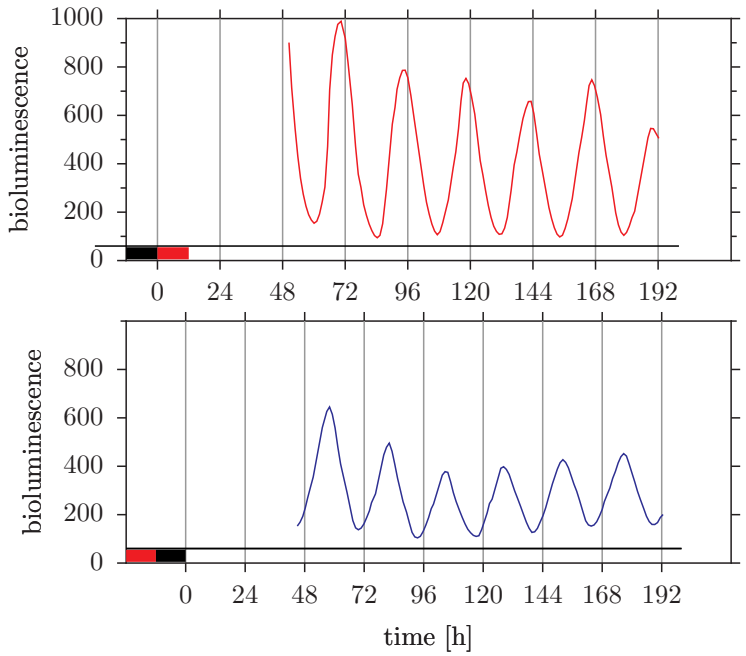

Figure 6.14: *Two cultures of* Synechococcus *were reared in 12:12 hour light-dark-cycles at* 30^0C *in such a way that one culture (red) was illuminated inversely to the other culture (blue). Therefore this culture entered continuous light 12 hours earlier (upper time axis). The bioluminescence oscillates in the cultures phase shifted by 12 hours. Under continuous light conditions this phase shift of the luminescence rhythm remains. After [809]*

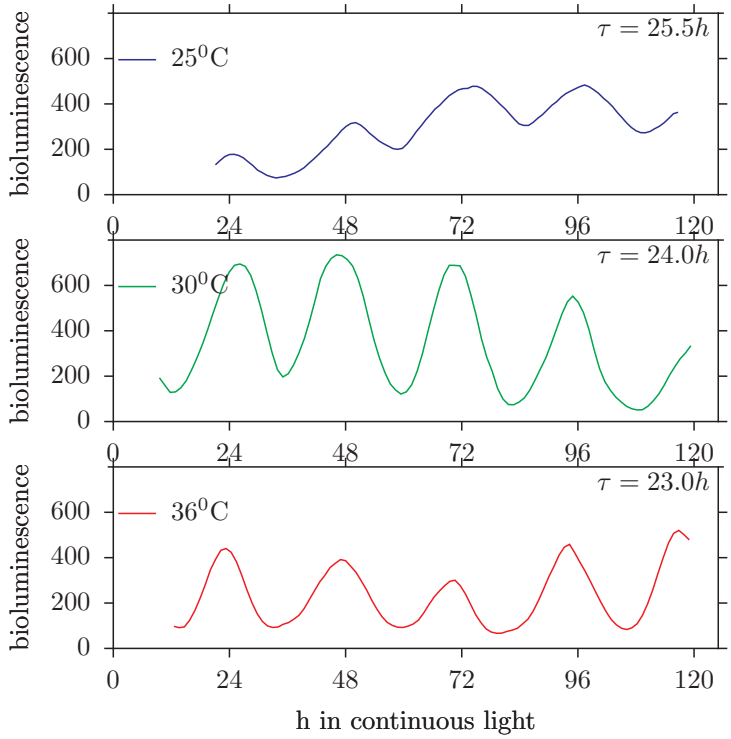

Figure 6.17: *Bioluminescence rhythm of transgenic* Synechococcus *cultures under continuous light (following 12:12 hour light-dark-cycles) at different temperatures of the sea water (upper curve: 25° C, curve in center: 30°C, lower curve: 36°C). The corresponding period lengths (25.5, 24.0 and 23.0 hours) differ only slightly, although the amplitudes are lower at lower and higher temperatures, respectively. The bioluminescence rhythm is thus temperature compensated. After [809]*

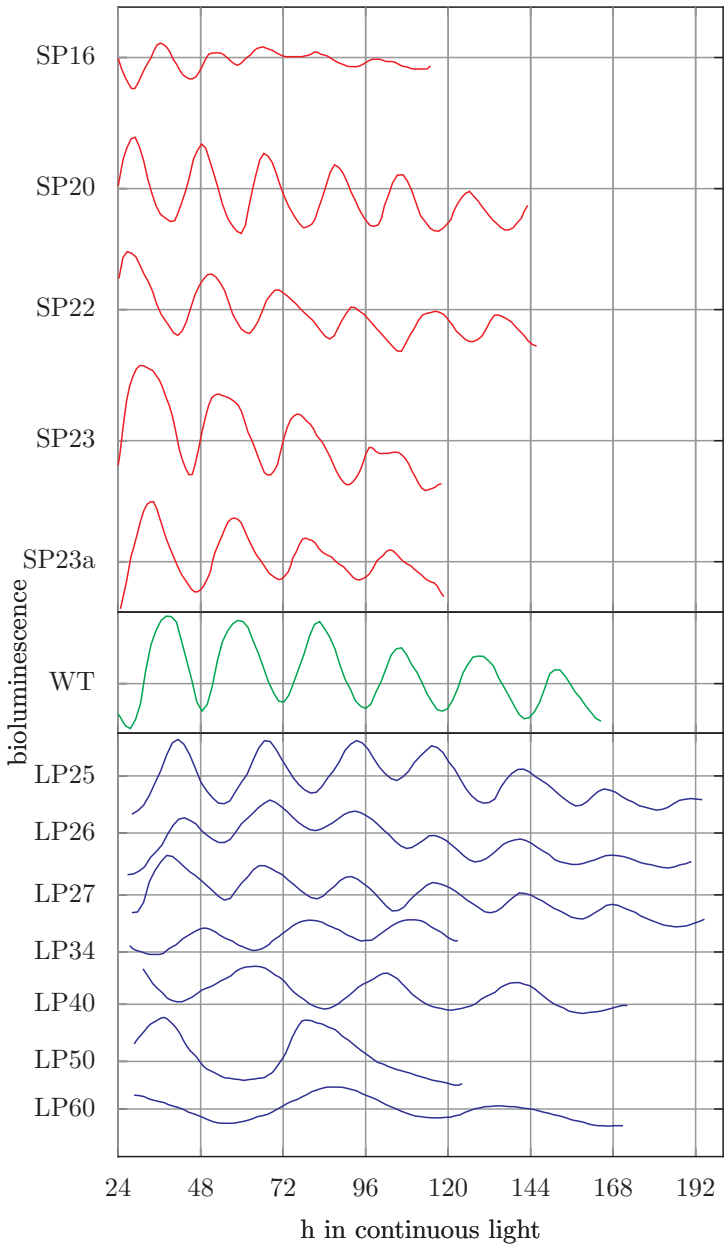

Figure 6.18: *Circadian rhythm of bioluminescence of transgenic* Synechococcus *('wild type', green curve) and of mutants with varying period lengths (from 16 to 60 hours length, red curves shorter, blue curves longer periods as compared to wildtype). After [810]*

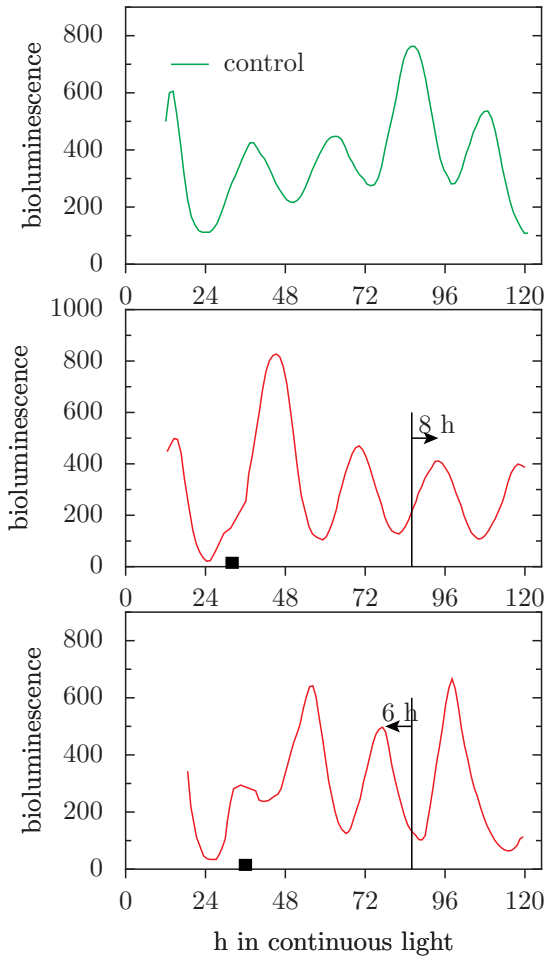

Figure 6.16: *Phase-shifting the bioluminescence rhythm of* Synechococcus *by dark pulses. Three cultures were kept in 12:12 hours LD-cycles and transferred at time 0 into continuous light. A single 4 hour dark pulse was administered 30 (center) respectively 34 hours (bottom) hours after onset of continuous light. The resulting phase shifts in respect to the untreated control are shown by blue arrows. After [809]*

be complemented by the wild-type-gene in order to re-obtain the period length of the wild-type. It turned out, that a gene *pex* had mutated. The intact *pex* gene increases period in the wild type ([838]). If a mutation disables its function, period length is shortened. The *pex* gene is not needed for normal growth. It is furthermore not essential for normal oscillations, since amplitude and shape of the rhythm are normal in the *pex* mutant. It is not yet known how the period is lengthened by *pex*. The structure of the gene product PEX is not clarified. The expression of the kaiABC genes (see later) is amplified by the *pex*-mutation. PEX seems therefore to suppress the expression of the kaiABC genes. In the outcome, the *pex* gene seems to modify the circadian clock somehow. At the same time its expression is modified by the circadian clock.

We will see (page 143) that the clock protein KaiA activates the KaiBC operon (PkaiBC). For this reason mutations were introduced into kaiA by PCR based mutagenesis. About 400 mutants were obtained ([1092]). In contrast to mutations in KaiB or KaiC the vast majority of these mutants showed lengthened periods up to 35 hours and rarely period shortenings. KaiA can change period directly or indirectly by lowering KaiBC expression. Two clusters of mutations with changed periods were found (residues 239-245 and residues 113-119). They represent important domains of KaiA for period determination. The very long period mutations are double mutations in kaiA. No arrhythmic mutations were found in this region. Regions of KaiA which sustain the rhythm by promoting kaiBC expression are located elsewhere.

KaiA interacts with the first (KaiCI) and the second (KaiCII) domain. Several mu-

tations have a reduced amplitude of the rhythm and some are arrhythmic. In the arrhythmic mutants sequences exist which enhance KaiBC expression. The arrhythmic mutants lack a start codon, have an inserted stop codon or possess a frame shift.

45 of the mutations were sequenced. 39 were point mutations, five showed overlap with other mutations. The results of these studies showed the crucial role of KaiA for sustaining the circadian rhythm in this cyanobacterium.

6.5 The clock work of the circadian system

How does the circadian clock of these prokaryots function?[1] Three properties help to find out how the clockwork functions: The ease to record the circadian rhythm, the clock mutants which were obtained, and the available molecular genetic techniques. The ways to do it are manifold and quite interesting, and much work is currently done on this line. It would take too much time and space in presenting them here. I therefore restrict this section to reporting some of the results of these studies.

Let us begin with a current model (figure 6.19). The clockwork is a feedback system where the products of three kai-genes[2] kaiA, kaiB and kaiC influence the transcription of their genes ([677]). The different kai products interact with each other in a way which is not fully understood.

According to [682] KaiA promotes the activity of a KaiC-autokinase. As a result KaiC is phosphorylated and degraded (figure 6.20). They also inhibit (KaiA-product) or promote (KaiC) the kaiBC-promoter[3]. A clock-output factor controls the kaiA promoter. In addition it controls genes ('clock controlled genes') which in turn lead to circadian expression of their products ([677]).

A number of experiments have shown, that the circadian control of gene expression can be specific for certain genes. They possess specific cis-elements and trans-acting factors. However, generally the circadian control of gene expression seems to be global. If the luxAB gene is inserted into other known regulatory regions, they show also a circadian rhythm in luminescence, although with weaker amplitude. With special methods the luxAB gene was inserted to all kinds of promoters. If the insertion was successful, the emittance of bioluminescence was circadian, irrespective of the particular promoter ([907], [503]). About 80% of these clones were maximally luminescent in the subjective evening[4]. Some clones showed maximal luminescence at other phases, some even at the subjective morning. Apparently there is a global circadian control of gene activity, and in addition also an individual control of specific genes with different phase positions (figure 6.21). It was proposed that the control of transcription with a gene-unspecific general mechanism might function for instance via 'super-coiling' of the DNA, energy charge (see page 359) or RNA-polymerase-activity. Without another control level, the particular gene would be timed by this unknown mechanism.

[1] für neuere Ergebnisse siehe http://w210.ub.uni-tuebingen.de/volltexte/2009/3993/

[2] kai means rhythm in Japanese

[3] kaiB and kaiC are both controlled by the kaiB-promoter

[4] that is at times where the light in a light-dark-cycle would go off; here the cultures are, however, under constant conditions

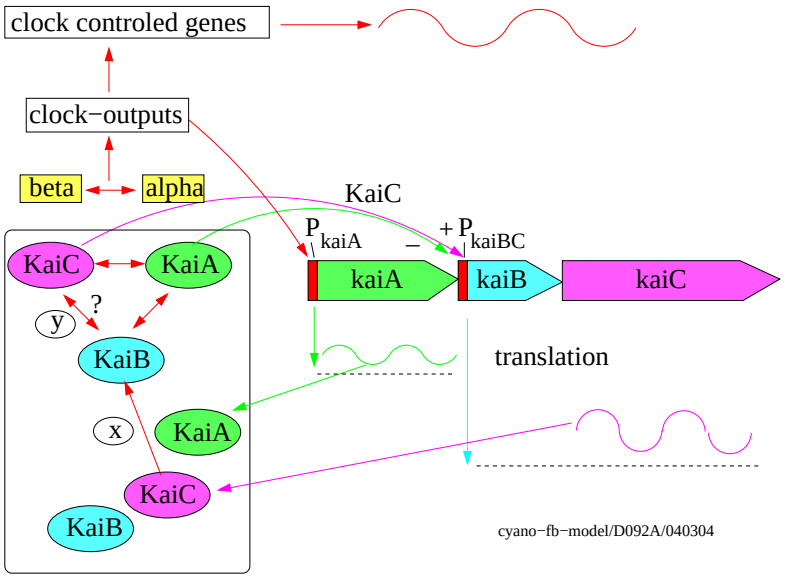

Figure 6.19: *Feedback model of circadian oscillator of* Cyanobacteria. *Three genes kaiA, kaiB and kaiC express rhythmically mRNA, and these produce the proteins KaiA, kaiB and KaiC. They interact with each other (box). KaiA inhibits the promoter P_{kaiA} (-). KaiC promotes the kaiBC-promoter (+). Time delays and feedbacks make up a circadian clock which influences clock-controlled genes in the output. Their products (red wave on top) are thus expressed in a circadian way. After [677]*

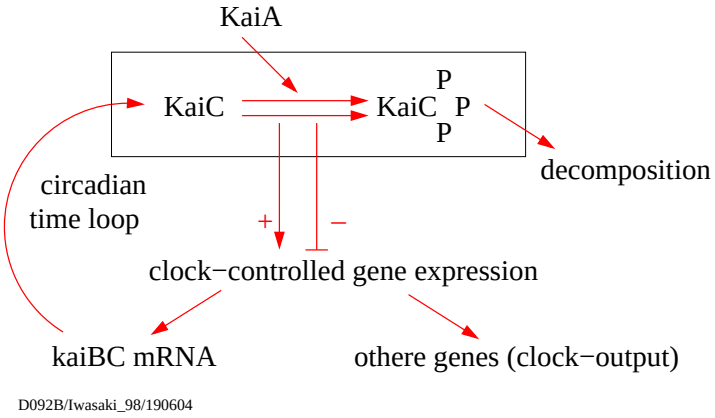

Figure 6.20: *KaiC is phosphorylated by a KaiC-autokinase. This process is activated by KaiA. The stochiometry of the* unphosphorylated *KaiC and the* phosphorylated *KaiC does perhaps determine, whether and how much the clock controlled genes express. After [677]*

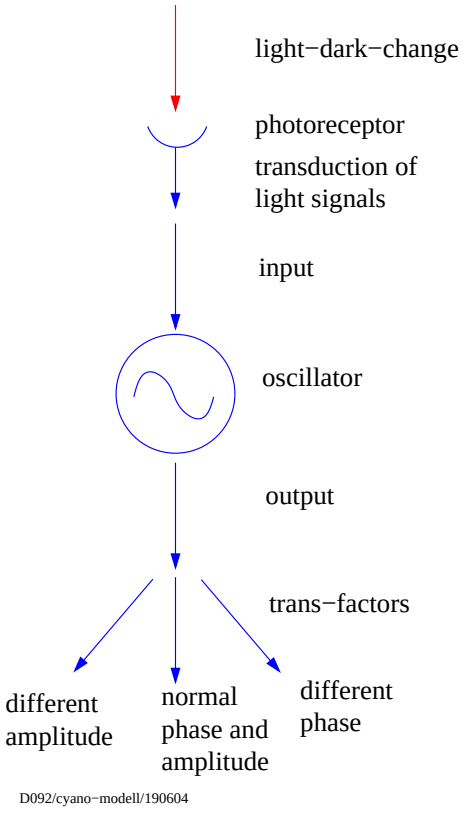

Figure 6.21: *Model of circadian gene-expression of* Synechococcus. *The circadian oscillator is synchronized by Zeitgeber such as the light-dark-cycle. One output of this oscillator influences transcription globally (normal phase and amplitude). Trans-factors affect amplitude and phase position of the rhythm additionally. After [677]*

It was also discussed, why in eukaryotic organisms only few circadian controls of gene activity were found ([503]). It might have to do with the way the circadian rhythm was discovered. In the eukaryotes studied so far a mRNA is identified as belonging to a 'clock controlled gene' only, if it varies a lot at different phases of the cycle. The method used in *Cyanobacteria*, however, reflects directly the activity of the promoters. The clock might thus be much more global, as originally assumed, but not all events in the cell oscillate in a circadian way.

Since *Cyanobacteria* are so strongly different from eukaryotes, it is questionable whether the circadian rhythm of the two groups is based on the same mechanism. According to the endosymbiont hypothesis, chloroplasts originated from *Cyanobacteria*. If circadian clocks were transferred in this way, the circadian system of plants and *Cyanobacteria* should be more alike as the clocks of fungi and animals are. If the functions of the participating genes are better known, this question might perhaps be answered. As it stands now it is more unlikely that circadian rhythms have been evolved from a common ancient mechanism. It is more likely that they have been invented several times at different stages of evolution (see page 402 in chapter 18).

6.6 Inputs of the clock

What about the inputs into the circadian clock? They must be synchronized with the periodically changing environment through time cues. We expect daily light-dark cycles, but also temperature cycles and perhaps other 24-hour rhythms of the environment to synchronize. Light-dark cycles and temperature

cycles do indeed synchronize the *Synechococcus*-clock. However, the time cues of circadian rhythms of *Cyanobacteria* are not yet intensively studied. In Cyanotheke the circadian rhythm is induced, when the medium of a stationary culture is diluted ([1333]). The sensors are not yet known. Red and blue light are the most effective wavelengths in synchronizing. In *Synechocystis* the photoreceptor is homologous to phytochrome ([742]).

The maxima of nitrogen fixation occur during the night, as mentioned already. Temperature cycles entrain also the rhythm (blue rectangles and curves). Under competing light-dark- and temperature cycles the light-dark cycle turns out to be the stronger time cue as shown in figure 6.22.

Other Zeitgeber of circadian rhythms of *Cyanobacteria* are not yet studied. In *Cyanotheke* the circadian rhythm is initiated if the medium of a stationary culture is diluted ([1333]). The sensors are not known.

6.7 Outputs

Circadian clocks control different events on the transcriptional, translational, biochemical and physiological level. In *Cyanobacteria* some of these events were already mentioned. Others are known, many wait for being discovered. It would for instance surely be worthwhile to study the movement of *Cyanobacteria* in respect to a circadian rhythm. Vertical movements which occur in some *Cyanobacteria* with the help of gas vacuoles, were already mentioned before. They should be checked for a circadian modulation more intensively.

Genes, which are not part of the circadian clock, but controlled by it, are for instance rpoD2. It codes a sigma 70 transcription factor[5]. RpoD2 seems to be a factor, which increases the amplitude of oscillations of some genes ([1490]).

In more than ten polypeptides of *Synechococcus* RF-1 a circadian control has been established ([663]). It is very likely that soon further components of the output of the circadian clock will be found. The control seems, however, complicated and not yet well understood. The global regulator-gene ntcA, which codes for a DNA binding protein NtcA, is a transcriptional activator of genes, which are under circadian control and have to do with the nitrogen assimilation ([142]).

6.8 Adaptive significance of circadian rhythms of *Cyanobacteria*

What is the adaptive significance of the circadian clock in *Cyanobacteria*? In nitrogen-fixing *Cyanobacteria* it takes care that photosynthesis and nitrogen occur at different times. This is important, since oxygen, which evolves during photosynthesis, inhibits nitrogenase as the key enzyme of nitrogen fixation. However, this partition in time does not seem to be necessary in all cases ([1114], [1247]). Diazotrophs can use quite different mechanisms to protect nitrogenase of oxygen (see [468] and [469]).

An important function of the circadian clock of the *Cyanobacteria* is probably 'warning of light'. The photosynthetic apparatus of *Cyanobacteria* is especially sensitive toward light-damage. Therefore its protection against light by events which

[5] transcription factors are made from 'switch genes´ and affect the transcription of other genes, for instance those which are involved in the clock mechanisms.

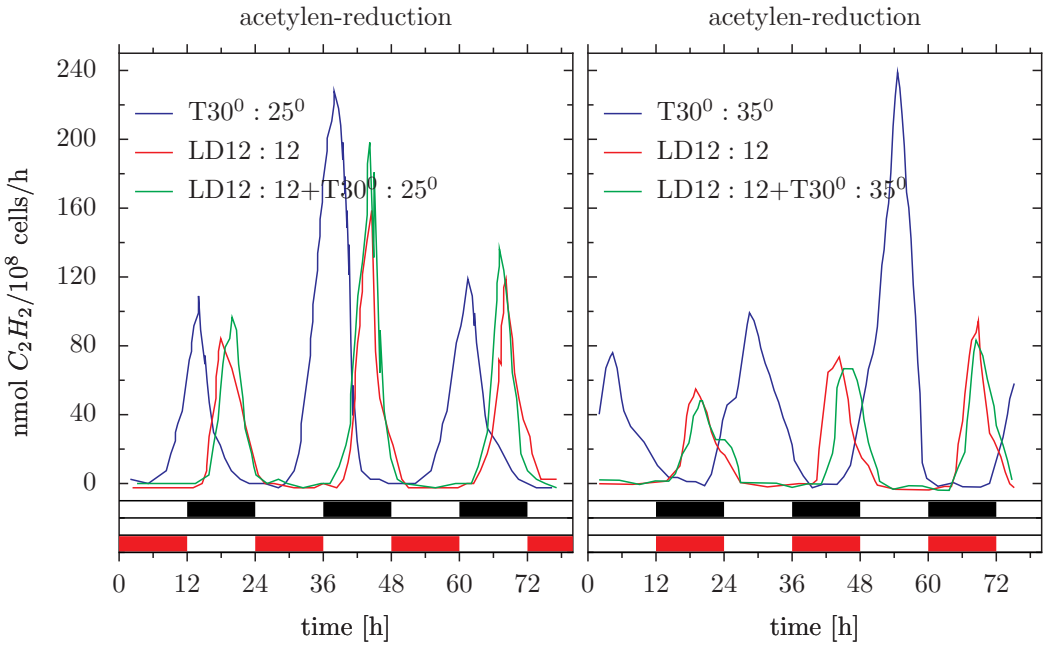

Figure 6.22: *Phase setting by light-dark- (LD, black rectangles) and temperature cycles (blue rectangles) in* Synechococcus *RF-1. The nitrogen fixation in LD 12:12 cycles (red curves, measured by acetylene reduction) occurs during the dark period (DP, black rectangles). The nitrogen fixation in temperature (T) cycles (blue curves) of 30°:25°C (left diagram) takes place during the later part of the high T and the first part of the low T period. In T cycles of 30°:35°C (right diagram) it occurs during the low T period. If high T and LP are given at the same time (left diagram), nitrogen is fixed during the DP and low T (green curve). If low T and LP are given at the same time (right diagram), nitrogen is fixed during the DP and high T (green curve). Thus the LD cycle is the main Zeitgeber in a situation where both, LD and T cycles are present either with low T during DP (left diagram) or during LP (right diagram). That is, the green curve (T and LD cycles) runs parallel to the red curve (LD cycles). After [898]*

are controlled by the circadian clock is of advantage.

It was shown that mutants with a shorter period length as the wild-type (the period of which is around 25 hours), are rapidly out-ruled in a 25-hour-day (12.5 hours light, 12.5 hours darkness) by the wild-type, whereas in a 22 hour-day the mutant supersedes the wild-type (see [703] and also page 397).

7 Circadian rhythms of CAM plants

As an adaptation to arid climates the Crassulacean Acid Metabolism allows plants to keep stomata closed during the hot day and take up CO_2 during the night. This reduces water loss considerably. Circadian rhythms are involved in the control of key enzymes of this metabolic specialty. The significance, biochemistry and regulation of the CAM rhythm will be presented as well as the recording methods. How light synchronizes the rhythm and which photoreceptors are involved is a further topic. Finally a model is explained which describes the biochemical mechanisms and its circadian control.

> The leaves of *Bryophyllum calycinum*, which on the whole have a herbaceous taste, are in the morning as acid as sorrel, if not more so; as the day advances, they loose their acidity, and are tasteless about noon and become bitterish toward evening. ([634])

CAM metabolism occurs in numerous plants. They belong to 33 families, such as *Cactaceae*, *Crassulaceae*, *Euphorbiaceae*, *Aizoaceae* (*Mesembryanthemum*), *Bromeliaceae*, *Asclepiadaceae*, *Orchidaceae*, *Liliaceae*, *Agavaceae*, *Asteraceae*, *Vitaceae* and *Geraniaceae*. Without orchids about 9000 species possess CAM; it is assumed that 7000 orchid species have to be added to this figure, which possess also CAM. These 16000 species make 6% of the angiosperms. It is likely that this special biochemical pathway evolved polyphylletically, since it is found in so many families. CAM occurs also in non-succulent plants, for example in *Tillandsia*. In this case of mesophyll-succulence only the photosynthesizing part is affected. The water plant *Isoetes* (*Lycopodiopsida*) possess also CAM.

Figure 7.1: *Cactus as an example of a CAM-plant from the Sonora-dessert in Arizona*

The CAM metabolism has its name (*Crassulacean Acid Metabolism*) from the *Crassulacea*. They are found in arid areas and possess succulent leaves and stems (figure 7.1). They are able to store water in water tissue (epidermis, subepidermis, for instance in *Peperomia*) or in parenchyma (mesophyll of leaves or cortex in the stem). The surface is reduced. This helps the plants to survive under dry conditions. In addition, the plants close their stomata during the day and open it in the night ([908]). CO_2 which is necessary for photosynthesis, is taken up during the night and stored as an organic acid (malate). In contrast to C4-plants, in which the CO_2-fixating cells are separated *in space*, in CAM-plants CO_2-fixation and -assimilation is separated *in time*. During the day malate is converted to CO_2 and ox-

alic acid. The carbon dioxide can now be used for photosynthesis.

A number of books and review articles is available in respect to the biochemistry, physiology and ecology of the CAM-metabolism ([795], [1115], [1477], [936], [1476], [1577]).

The CAM-metabolism is controlled by a circadian clock and is therefore presented here. Recent reviews are [1565], [1577], [205].

7.1 Examples, properties and significance of CAM

Whereas a C3-plant needs 0.5 to 1 liter of water in order to produce 1g of sugar, a CAM-plant needs only 0.5 to 0.6 liter if well watered, 0.18-0.5 if less well watered and 0 at poor water supply. The payoff is a slower growth. C4-plants need 0.25 to 0.35 -liter, but grow faster as CAM-plants.

CAM-plants possess in their photosynthetically active tissue large intercellular space. This facilitates gas exchange. CAM cells are large with thin walls and a small peripheral cytoplasm. They contain relatively few chloroplasts with and without grana and often rich in starch. The vacuoles of the chlorophyll-containing cells are large and store among others malate.

CAM plants possess fewer stomata as compared to other plants. In *Kalanchoe* there are about 3200 stomata per cm^2 on the upper side of the leaves and 4700 on the lower side. The opening- and closing mechanism of stomata of CAM-plants is the same as in other plants. Osmotically active substances accumulate under energy consumption, the turgor increases and water is taken up. The guard cells swell, the stomata open. Stomata are, however, not necessary for CAM-metabolism to occur. In the water plant *Isoetes* (*Lycopodiopsida*) stomata are lacking; in spite of it this plant possess CAM. It is induced as in other water plants by the low CO_2-content of the water during the day.

The intercellular spaces of CAM-plants are larger as those of other plants. Often a sub-epidermal water tissue is present in addition to the epidermis cell layer. The cuticle of CAM plants is less water-permeable. The tissue of CAM plants warms up more slowly and cools down more slowly. Growth occurs even at 0^0 and 50^0 C. The heat resistance fluctuates in a circadian pattern.

The CAM metabolism is, depending on the plant species, facultative or obligatory. *Mesembryanthemum crystallinum* is an example for a facultative CAM-plant. It changes from a C3-plant to a CAM-plant, if the soil contains much salt (200 to 500 mM) or is dry ([363]). In the Mediterranean the plant germinates in January at the rainy season. It grows first as a C3-plant, and with the onset of the dry season in July the metabolism switches to CAM. In other plants the CAM is induced photoperiodically (*Kalanchoe blossfeldiana*) and/or thermoperiodically.

As far as the ecology, productivity and economic significance of CAM-plants is concerned, see [795], [937], [1577].

7.1.1 Significance of the vacuole for CAM

CAM plants contain usually cells with a large central vacuole. The cytoplasm takes only 1% of the cell volumes, the cell wall 2% ([1424]). The vacuoles are important for osmotic events, ions storage and -transport and the regulation of the CO_2-metabolisms. The osmotic pressure is

low, partly less than 1 MPa. This is true also for plants with a high water deficit. The cell walls are elastic and can be used by the plant as a water reservoir.[1] Besides malate, citric acid and isocitric acid is stored in the vacuoles. Malate can be used as an osmotic to 'harvest water' ([1394], [1274]). A pH-stat-mechanism keeps the pH in the cytoplasm constant ([293]). Furthermore an equilibrium between anions and cations is adjusted.

7.2 Biochemistry of CAM

During the CO_2-fixation **in the dark** the following steps occur:

$PEP + CO_2 \Rightarrow (PEP - Carboxylase)) \Rightarrow OES \Rightarrow malate$[2]

The acids are formed, depending on the plant, either from polysaccharides (starch, glucan: *Kalanchoe daigremontianum*) or from water soluble hexoses (*Ananas*, *Clusia*).

CO_2 reacts probably as HCO_3. Malate is during the night actively transported into the vacuole and leaves the vacuole passively, to be used during the day in the cytoplasm while photosynthesis occurs. Light (far red!) stimulates the efflux of malate from the vacuole into the cytoplasm ([1059]). Malate is decarboxylated in the cytoplasm by the malate-enzyme and the CO_2 given off used for photosynthesis (figure 7.2).

[1] A high malate content leads to water influx into the vacuole. This would change turgor. However, since no pressure changes can take place across the tonoplast, the membrane thickness changes (1/100 bar changes already the thickness by a few Angstrom ([1613])). This in turn changes the membrane properties such as the permeability strongly.

[2] Malate can be converted into other acids such as aspartic acid

The following enzymes participate in CAM:

- PEPCase is an allosteric enzyme and very active. It is present in a day and a night form.[3]
- Glucose-6-phosphate activates PEPCase.
- Malate dehydrogenase MDH converts oxalic acid in malate by reducing $NADH_2$ to NAD^+. MDH is activated by light.
- Malate enzyme decarboxylates malate to pyruvate by converting $NADP^+$ in the presence of Mg^{2+} to $NADPH_2$. A 55kDa- and a 61 kDa subunit and a dimer with a lower and a tetramer with a higher affinity for malate exist.
- PEPCkinase converts oxalic acid to PEP and CO_2, whereby ATP changes to ADP. At high malate enzyme-activities the PEPCkinase is inhibited, at low activities activated.

PEP is formed from glucose-phosphate. This is done by phosphorylation of starch or other polyglucanes (figure 7.3). At high CO_2-uptake malate accumulates, at low CO_2-uptake it dissipates. At the end of the dark period malate has accumulated in the vacuoles (up to 200 mM) and the leaf cells are strongly acid. More than 90% of the malate has accumulated in the vacuole.

During the **light period** malate flows from the vacuole into the cytoplasm and is decarboxylated by the malate enzyme to pyruvate. CO_2 is assimilated in the

[3] PEPCase is coded by the gene Ppc1 and Ppc2 ([275]). The night form is a tetramer, the day form a dimer. PEPCase of C3 plants differs from that of CAM plants.

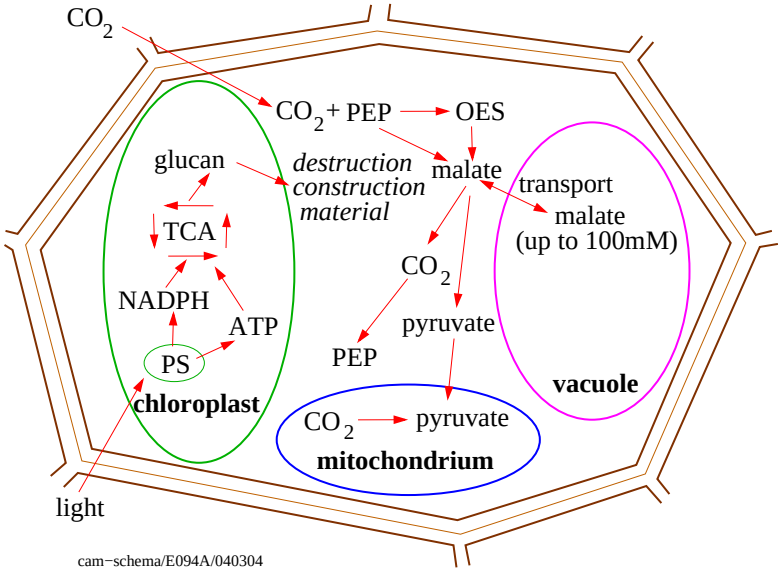

Figure 7.2: *Scheme of the CAM of a cell. In darkness (blue) CO_2 is with the help of PEPCase fixed to PEP and OES formed. This is converted to malate. It is transported into the vacuole. If the concentration has reached 100 mM or if the light period begins, malate flows out of the vacuole. It is decarboxylated by the malate enzyme to pyruvate. The CO_2 which is released is used in the chloroplasts during photosynthesis and converted in the tricarbonic acid cycle (TCA) into starch or other polyglucanes. Glucanes serve as construction material and are the source for PEP. After [794]*

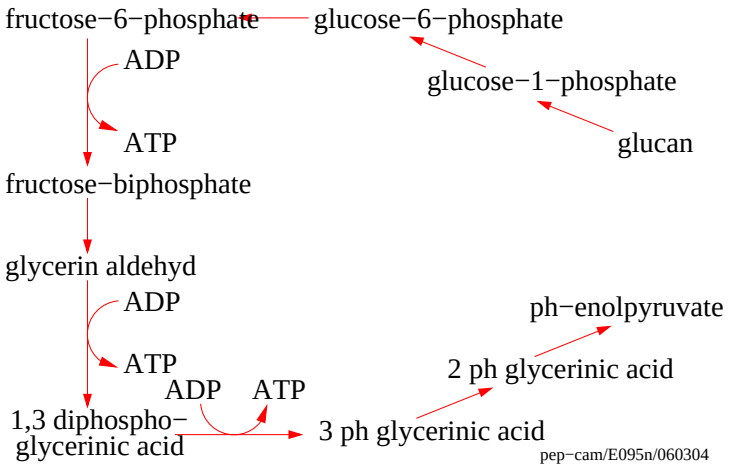

Figure 7.3: *Phosphoenol-pyruvate formation from glucan via glucose phosphate, fructose-phosphate, glycerin aldehyde and glycerine acid. After [1435]*

light during the course of photosynthesis and carbohydrates are synthesized. At the end of the light period all malate is broken down for photosynthesis and the concentration is as low as 30 mM.

7.2.1 Compartmentation of the CAM enzymes

The PEPCase is membrane-bound, but is not present in chloroplasts, mitochondria and microbodies. MDH is in the membranes of mitochondria and microbodies, malate enzyme in mitochondria, in cytosol and in the chloroplasts. The products of the CO_2-dark fixation are also compartmented and separated from mitochondria, microbodies and chloroplasts. There are possibly isozymes in the different compartments.

7.2.2 Regulation of CAM

The CAM metabolism is strongly regulated both by environmental conditions and by metabolic events. Thus, environmental temperature, light and water content of the soil and the air influence CAM. CAM is furthermore photoperiodically controlled (*Kalanchoe blossfeldiana*) ([795]). Internal factors are stomata, enzymes, water conditions and transport of substances into the compartments. CO_2-free air inhibits malate synthesis during the night, whereas CO_2-enriched air promotes malate synthesis.

During the dark period malate is stored in the vacuole. If the storage capacity of the vacuole is exhausted, the malate synthesis by PEPCase in the cytoplasm is inhibited due to an increase in malate concentration in the cytoplasm (substrate inhibition). This negative feedback control is rather common in the metabolism.

Furthermore the CAM-metabolism is also controlled by the circadian clock: Malate concentration, PEPCase- and MDHase-activity fluctuate in a circadian pattern. The circadian control is post-translational and affects the specific activity of PEPCase (and not the synthesis and/or degradation). PEPCase occurs in a day- and a night form. The night form is the active one, the day form is inactive: It is ten times more sensitive to malate-inhibition (K_i malate 0.3mM) as compared to the night form (K_i 3.0mM). The specific activity under V_{max} is, however, constant. Phosphorylation of a serine residue transforms the day form of the PEPCase into the night form. Dephosphorylation leads to the day form ([1089], [1088]). In this way it is prevented that malate is decarboxylated during the day in a 'futile cycle' by malate enzyme. The changes occur already 1-2 hours before onset of light and 4-6 hours after onset of darkness. This transformation occurs also under continuous light. It is therefore under circadian control.

PEPCase is phosphorylated by a PEPC-kinase and dephosphorylated by a PEPC-phosphatase. It was shown that synthesis and destruction of the PEPC-kinase are responsible for the CO_2-rhythm of CAM plants ([1565] and figure 7.4).

7.3 Recording

CO_2 is usually recorded with an infrared-absorption recorder (URAS) (figure 7.5). The temperature is kept constant in the cuvette and water removed from the air, since it absorbs infrared light in the same way as CO_2, which would falsify the recordings.

Newer methods use the O_2/CO_2 -

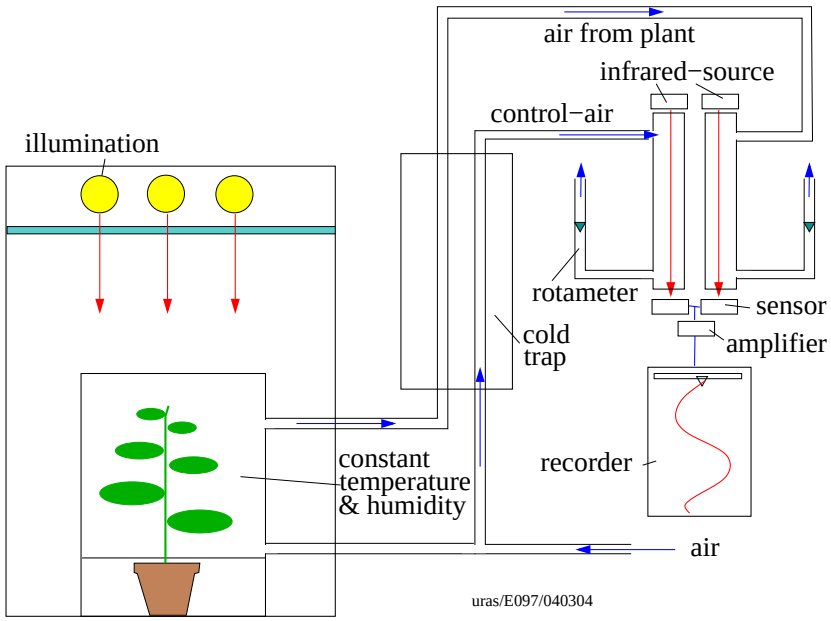

Figure 7.5: *Recording of CO_2 of CAM-plants with an infrared -absorption recorder (URAS). The plant is kept in a cuvette with constant temperature and humidity. An illumination box allows controlled illumination at different times. An air stream passing the plant is freed in a cold trap from water and passes an URAS. Depending on the CO_2-content different amounts of infrared-light are absorbed. Parallel to these measurements the CO_2-content of the control air is recorded and from the difference between these values the CO_2-content of the air passing the plant is calculated and recorded. The air stream can be controlled with a rotameter. After [794]*

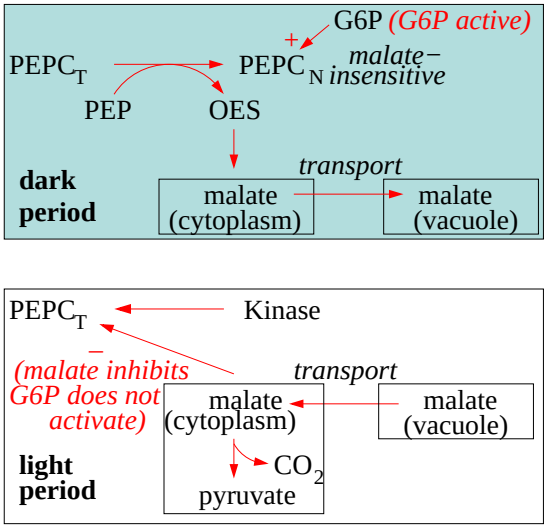

Figure 7.4: *Significance of PEPC-kinase in CAM-plants for the phosphorylation of PEPC and for the CO_2-rhythm. During the dark period the day form PEPC ($PEPC_D$) is converted by a phosphatase into the night form ($PEPC_N$). The pH and the malate concentration play a role. Glucose-6-phosphate promotes this conversion. The phosphatase does not show a rhythm of its activity. The night form is insensitive against inhibition by malate as compared to the day form. During the light period a kinase produces the day form of the PEPC ($PEPC_D$). The kinase is influenced by the circadian clock. The day form is strongly inhibited by malate, Glucose-6-phosphate does not activate the day form. After [206], [1584] and [1088]*

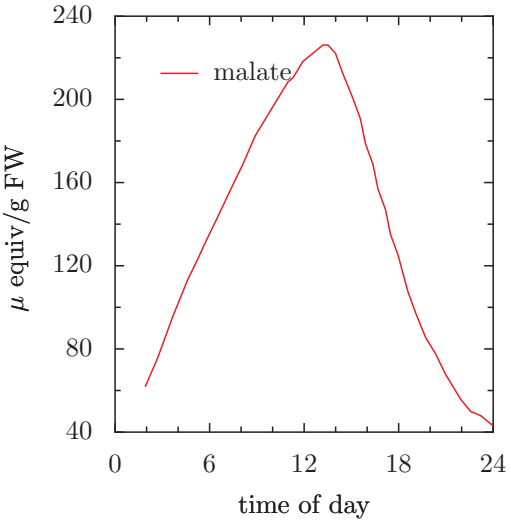

Figure 7.6: *Changes of the malate content (µequivalents per g fresh weight) in the light dark cycle of a day in* Kalanchoe tubiflora *(after [1435])*

exchange, and the isotopes ^{18}O and ^{13}C are determined in a mass spectrometer. ^{14}C - labeling can be used also for recordings. Furthermore the conductivity of the leaves can be recorded. It fluctuates with the malate content in a circadian way.

7.4 CAM in the light-dark-cycle and under constant conditions

In light-dark cycles malate accumulates in the vacuole during the dark period. As soon as the light period begins, the malate content decreases rapidly and strongly (figure 7.6). Malate is broken down in the cytoplasm and the formed CO_2 is synthesized in the Calvin-cycle to carbohydrates in the course of photosynthesis. Which steps occur during these events is the content of the following section.

Under constant conditions PEPCase as

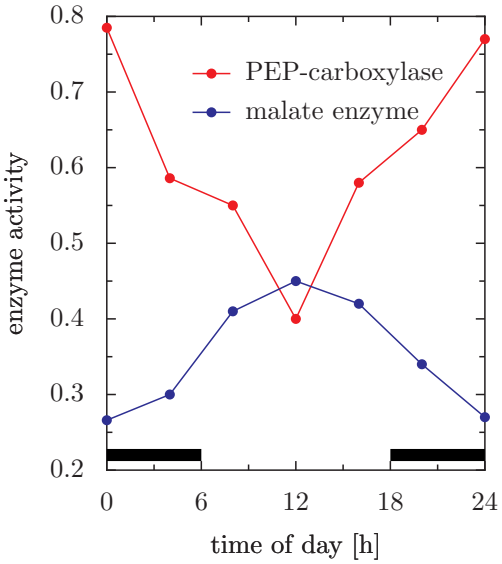

Figure 7.7: *The activity of the PEPCase and of the malate enzyme have an inverse course to each other. PEPCase is strongly active in the onset of the night, malate enzyme in the late night and in the morning. Enzyme activity in units per 100 mg fresh weight. After [795]*

well as the malate enzyme are fluctuating in a circadian rhythm (figure 7.7). The phase relationship of the two rhythms differs ([1193]).

In continuous darkness with CO_2-free air the CO_2-delivery of respiration is circadian, with low values during the subjective night and high values during the subjective day (figure 7.8, [1565], [1561], [1193]). The rhythm is observed for 2-3 cycles only and is strongly damped. The period length of the rhythm is between 10 and 28^0C relatively independent of temperature ($Q_{10} = 1.03$) (figure 7.8). With higher temperatures period is somewhat shorter. If every 24 hours a red light pulse is given for 15 minutes, the rhythm does not damp out.

The CO_2-uptake **in continuous light** in normal air is circadian (shown with labeled CO_2). The rhythm is not much damped and can be observed, depending on the light intensity, 10 days and longer (figure 7.8). The malate content of the vacuole is lower, because the tonoplast is permeable for malate in the light. The temperature compensation of this CO_2-rhythm is less precise as that in continuous darkness ($Q_{10} = 0.8$) (figure 7.8). The period increases from 16 hours at 10^0C to 24 hours at 32^0C.

Photosynthesis does not fluctuate in continuous light. However, transpiration, conductivity of the stomata and the CO_2-partial pressure do show a circadian rhythm. The CO_2-uptake is determined by the capacity of the mesophyll cells for carboxylation.

Without epidermis the rhythm in the mesophyll disappears. It is concluded, that the rhythm is brought about by circadian changes in conductivity of the stomata.

Below 10^0C and above 30^0C or at 5% CO_2-content of the air the rhythm disappears. The system stays in a fixed point for low respectively for high temperatures, both of which are phase shifted against each other by 180^0.

The phase of the rhythm can be shifted by light and temperature-pulses. Red light acts as white light, blue light has no effect ([1561]).

The **photoreceptors** have been studied. It was found that the phytochrome system is responsible for the perception of light in the CAM-rhythm. It influences channels and pumps, perhaps via IP_3, protein kinases, Ca^{2+}-channels and turgor (see subsection 20.13.1).

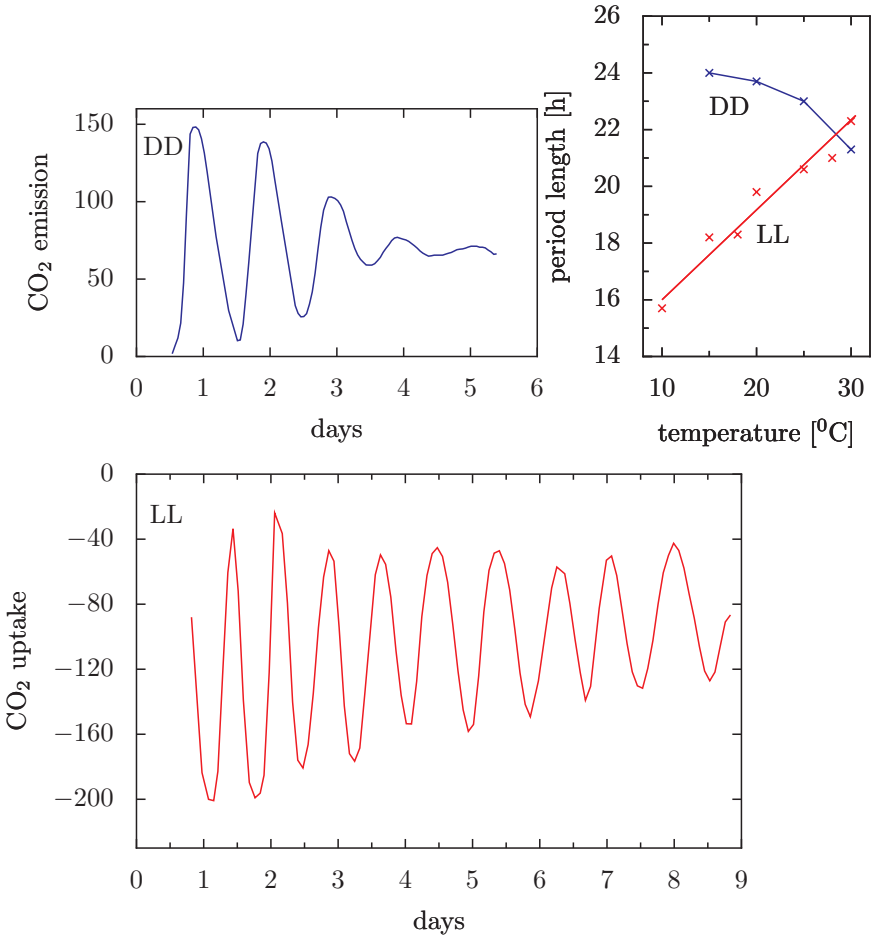

Figure 7.8: *Damped CO_2-rhythm of a CAM-plant under continuous darkness at 15°C, added air without CO_2 (top curve left). CO_2-delivery of a CAM-plant in continuous light, added air with CO_2 (lower curve). The y-axis shows the amount of delivered (upper curve) respectively of taken up CO_2 in µg per g fresh weight and hour. After [1561], [1563]. The low temperature dependency of the period length of the CO_2-rhythm in continuous darkness and the somewhat stronger temperature dependency of the period length of the O_2-rhythm in continuous light (upper right curve) is illustrated. After [14] and [1562]*

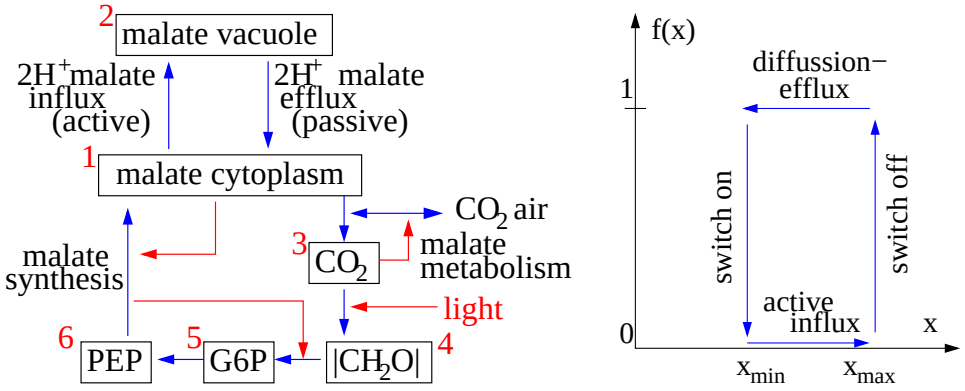

Figure 7.9: *a: Six storages (red numbers of boxes of cytoplasm (1), vacuole (2), CO_2-storage (3), starch-(4), glucose-6-phosphate (5) and phosphoenol-pyruvate-storage (6)) are connected with each other by fluxes (blue arrows). Feedback of the malate in the cytoplasm (red bended arrows) upon the malate synthesis, of the malate synthesis upon the starch conversion into glucose-6-phosphate and of the CO_2 in the plant with the CO_2 in the air in connection with a hysteresis switch (right figure) for the malate-efflux out of the vacuole lead to oscillations. For the regulation of the malate-influx and efflux of the vacuole a hysteresis-switch (b) plays an important role. The hysteresis delays the switching and leads in this way to the long periods of the circadian rhythm. After [938]*

7.5 Models

Models were proposed which explain the circadian rhythm in the CAM ([938] and figure 7.9). Six storages (boxes cytoplasm, vacuole, CO_2-storage, starch-, glucose-6-phosphate and phosphoenol-pyruvate-storage) are connected with each other by fluxes. Feedback of the malate in the cytoplasm upon the malate synthesis, of the malate synthesis upon the starch conversion into glucose-6-phosphate and of the CO_2 in the plant with the CO_2 in the air in connection with a hysteresis switch for the malate-efflux out of the vacuole lead to oscillations. Decisive is the hysteresis property of the tonoplast membrane ([1063]). Malate enters actively the vacuole. At a maximal malate content of the vacuole the properties of the tonoplast change. The active influx stops, malate leaves the vacuole. The malate concentration in the vacuole has to reach a low value x_{min} until malate can enter the vacuole again. This switch between active and passive malate transport has also hysteresis-properties. The delay in switching is responsible for the time delay, and this in turn for the long circadian period. For more details and more recent publications see [111] and [112].

8 Flower clock *Kalanchoe*

The flowers of Kalanchoe blossfeldiana *open during the day and close during the night. How can one record it? Which structures and mechanisms are responsible for it? What kind of models can be used to describe these events? This will be the content of this chapter.*

Kalanchoe blossfeldiana is endemic to Madagascar. It belongs to the family of the *Crassulaceae*, which are in the order of *Saxifragales* and the subclass of *Rosidales*. Crassulaceae are cosmopolitans. They are found in all warm, arid, rocky areas with long dry seasons. The red flowers of *Kalanchoe* are arranged in whorls (figure 8.1). The seeds are tiny. Flower formation is induced by short day. Under long days the plants do not flower, but stay in the vegetative state (see subsection 13.2.5).

The flowers of *Kalanchoe* are open during the day and closed during the night (figure 8.2). This movement continues under constant conditions of weak green light. The period length of the rhythm is about 22 hours. The rhythm is therefore driven by a circadian clock. If flowers are cut off the plant and mounted on a suitable solution, the circadian rhythm of opening and closing continues.

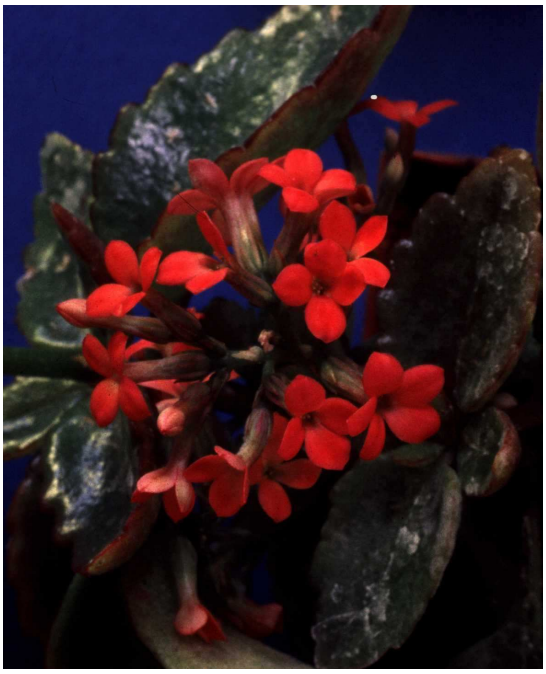

Figure 8.1: Kalanchoe blossfeldiana *induced by short day treatment to flower. During the day the petals of the flowers are open*

8.1 Anatomy of the flowers and mechanism of petal movement

The flowers consist of a calyx, four petals which are fused in the lower part forming a flower tube, and the carpels with

pistil and stamina. The petals consist of an upper epidermis with papilla like cells and red colored vacuoles (figure 8.2). The lower epidermis consists of a pavement epithelium. Between the two epidermis layers there are about 15 layers of parenchyma cells. They are responsible for the movement of the petals and are therefore called motor cells. In the interior of the petals conductive and supporting tissue form a central cord.

The opening and closing of the petals is due to turgor changes in the motor cells (figure 8.3). It is likely, that the same processes occur as in the joints of legume plants. However, the anatomy of the *Kalanchoe* flowers is simpler. There is only one uniform region where the movement occurs, no special joints with extensor and flexor are formed. The lower epidermis serves probably as a deformable structure on top of which the expanding and shrinking motor cells of the parenchyma are arranged. The upper epidermis allows by its very structure of its papilla cells to follow the extension of the motor tissue. Turgor changes in these papilla cells have been demonstrated.

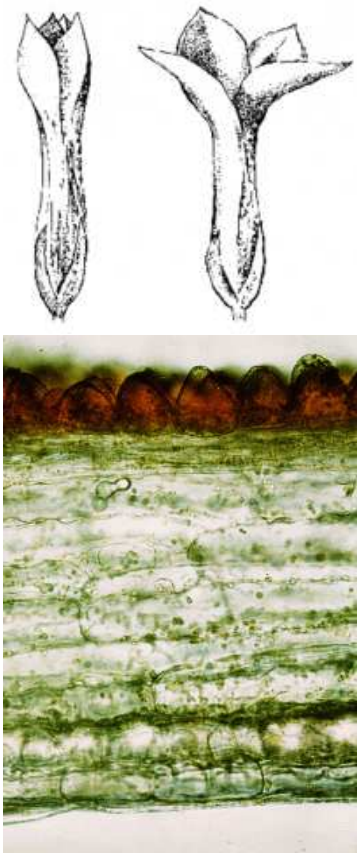

Figure 8.2: *Flower of* Kalanchoe blossfeldiana *with closed (left) and opened petals (right). A cross section through a petal (central part of the figure) shows papilla like upper epidermis cells. They are colored red by anthocyanin in the vacuoles. Underneath are several layers of parenchyma cells. The lower epidermis is a pavement epithelium (lower part of the figure)*

The physiological basis of the movements were studied by [1336]. The K^+ and Na^+ concentrations in the flowers change during the day (in *Albizzia* and *Mimosa* the Na^+ concentration does not change). Ca^{2+} however stays constant. The turgor rhythm is shifted in respect to the water uptake rhythm by more than 3 hours ([1620]). The two processes are thus independent of each other. This is also shown by independent reactions of single petals of a flower if a phase shifting light pulse is illuminating just one of the petals.

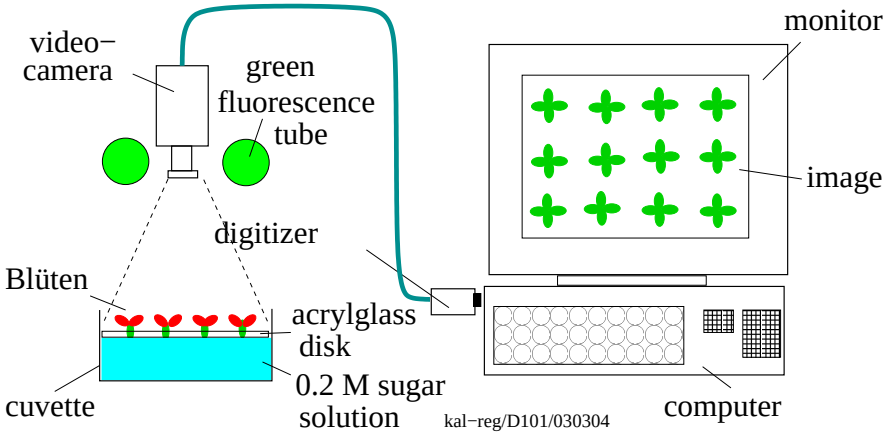

Figure 8.4: *Recording of the* Kalanchoe *petal movement rhythm with an imaging system. The flowers are plugged off the whorl with a pair of tweezers and mounted in holes of an acrylglass disk. This disk floats on a 0.2M sugar solution. A video-camera takes pictures of the flowers in regular time spans under continuous green light. The images are digitized and with the help of a program evaluated on an Atari-computer. Depending on the situation the rhythmic movement of each individual flower or of the average behavior of all flowers or of both is recorded*

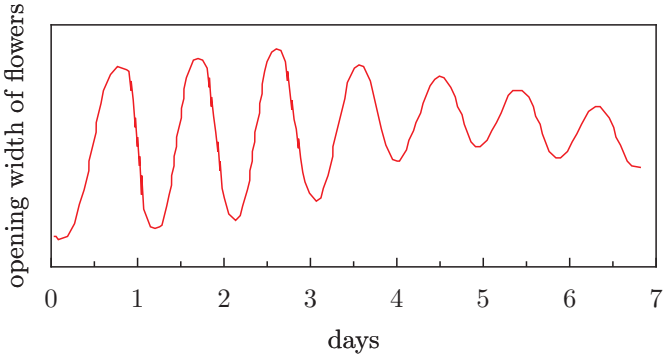

Figure 8.5: *Petal movement of a single and isolated* Kalanchoe *flower measured with an imaging system as shown in figure 8.4. Opening width of petals after transfer from a light-dark cycle of 12:12 hours into continuous weak green light*

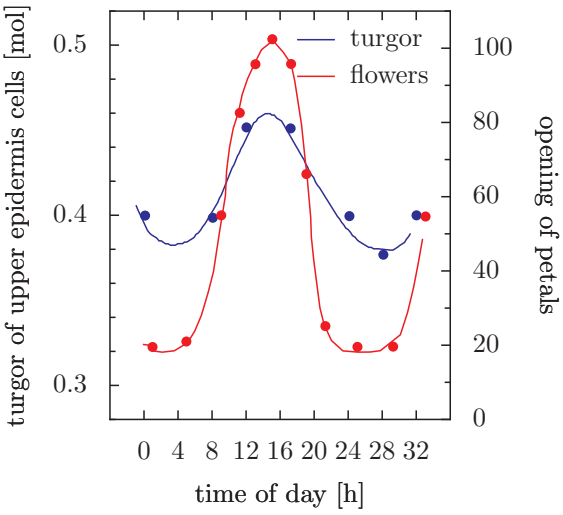

Figure 8.3: *Time course of the suction force in the upper epidermis cells of the petals (red) and petal movement (blue) in* Kalanchoe blossfeldiana. *After [1337]*

8.2 Recording of the petal movement

Different methods were used in order to record the petal movement of *Kalanchoe*. Originally photo-electric methods were used. The shadow of the flowers fell on solar cells. Depending on the degree of opening of the flowers the electric signals differed and could be recorded with voltage recorders or computers and the curves plotted as a function of time. Lately imaging analysis methods have been used. They are easier to use and a large number of flowers can be recorded simultaneously. The data can be analyzed directly on a computer using time series analysis programs (figure 8.4). The resulting curves of the petal movements are very smooth (figure 8.5) and the period lengths can be determined easily by eye or using time series analysis methods.

8.3 How light influences the petal movement

In the following the effect of continuous light, of continuous darkness and of light pulses on the *Kalanchoe*-petal movement is described.

In contrast to continuous darkness continuous light damps the rhythmic movement of the *Kalanchoe*-flowers. The period length depends on the light intensity and the wavelength ([746]).

Light pulses shift the rhythm, if offered at different phases of the cycle (figure 8.6). The phase response curves belong to the weak or strong type, depending on the strength of the pulse ([718]). The outermost part of the petal is especially sensitive to phase shifting light pulses, the basis of the petals does not react. The petals react independently from each other to light pulses. 9erg/cm^{2}sec of red light (632nm) is not shifting, 90erg/cm^{2}sec shift phase.

An action spectrum shows, that at least two different pigments are involved, one with a maximal effect in the red spectral region between 600 and 650 nm, another one with a maximum in the UV-part of the spectrum ([1335]). Phytochrome might be involved (figure 8.7), although the effect of red light is not reversible with far red (discussed by [1335]). A similar situation was described by [1516] and [315] in the unfolding of grass leaves. It is known that different phytochromes are used for different tasks in the plant. One of the phytochromes has a short term effect, another one is formed later, accumulates and has a long term effect. Phytochrome is the receptor of light effects also in other circadian rhythms (see subsection 20.13.1). In other cases blue-absorbing pigments are responsible (example: *Coleus*, [548], but

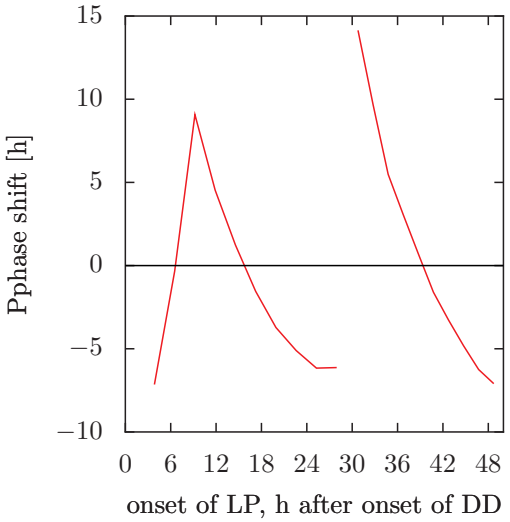

Figure 8.6: *Phase-response-curve of the circadian petal movement of* Kalanchoe *to a three hour light pulse, given at different times after the onset of 'continuous darkness' (weak green light). Advances of the rhythm in respect to the control are plotted upward, delays downward (in hours). After [718]*

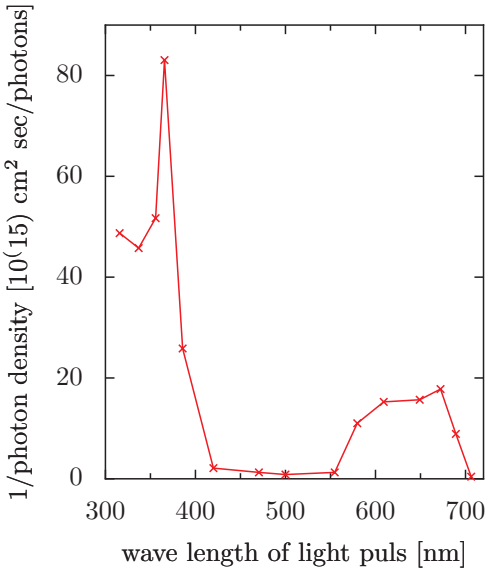

Figure 8.7: *Light pulses of different wavelengths and photon densities were applied to Kalanchoe flowers in order to shift the rhythm of the petal movement. The photon density needed to shift the rhythm by 3 hours was determined for different wavelength (x-axis). Since low photon densities are needed at the most effective colors UV- and red, the reciprocal was used on the y-axis. The resulting action spectrum indicates, that at least two different pigments are involved. After [1335]*

[1564]). Phytochrome is, as are other pigments, not an essential part of the circadian oscillator. In *Albizzia*, *Phaseolus* and *Mimosa* it is localized in the joints. In *Kalanchoe* screening pigments are also involved (anthocyanin). The papillae of the upper epidermis increases the efficiency of light absorption ([545], [974], [1519]).

8.3.1 Point of singularity

There is another effect of light on the petal movement rhythm of *Kalanchoe*. If a very special pulse of light is adminis-

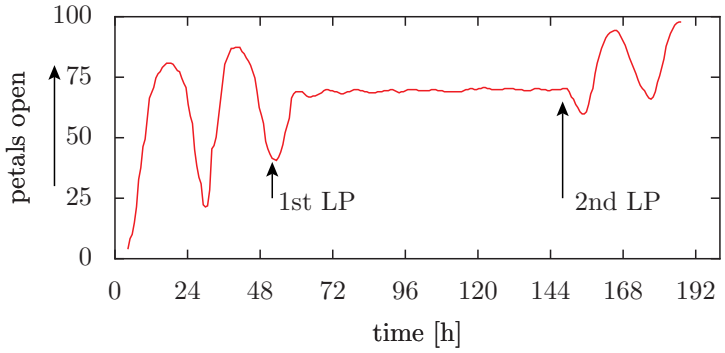

Figure 8.8: *Arrhythmia is induced by a special treatment of the* Kalanchoe *flowers with a particular light pulse. If a light pulse LP_1 is administered at a phase where the flowers are closed, and if the duration and intensity of the light pulse is 'the right one', the petals stop moving. The system has become arrhythmic. A second light pulse LP_2 of the same duration and intensity induces the system to oscillate again. After [386]*

tered at a certain phase, the rhythm is stopped. A red light pulse of 120 minutes and 2300erg/cm^{2}sec given 0.5 hours before until 0.5 hours after the third minimum induces arrhythmicity in many of the flowers (figure 8.8). The same effect is shown by using 60 minutes of UV light of 3600erg/cm^{2}sec. A second light pulse or a temperature pulse or even certain substances administered as a pulse re-initiate the petal movement rhythm. Other substances might be without effect. Using this method one can find out whether a substance interacts with the state variables of the oscillating system. State variables are important parts of the oscillating system which are needed for the oscillator to function. Only if a substance interacts with one of the state variables the arrhythmic flower will start to oscillate again ([386], [379]).

8.4 Effects of temperature

Different but constant environmental temperatures influence the period length of the *Kalanchoe*-petal movement only slightly. Only the phase relationship is altered: At high temperatures the first maximal opening is earlier as at lower temperatures. Below 13^{0}C and above 30^{0}C the rhythm is strongly damped or disappears ([1111]).

In contrast to constant temperature, temperature pulses influence the *Kalanchoe* clock in a similar way as light pulses do. Phase response curves were constructed both for heat pulses and for cold pulse (figure 8.9 and [382]). Pulses with increased temperature act like light pulses. Temperature influences also the time it takes for a light signal to reach the oscillator ([383]).

8.5 Effects of substances

Kalanchoe-flowers are well suited to check the effect of different substances on the rhythmic movement of petals, since they can be cut of the plant and put into a solution to which the substance can be added.

In water the oscillation is damped. If sugar is added to the water the damping

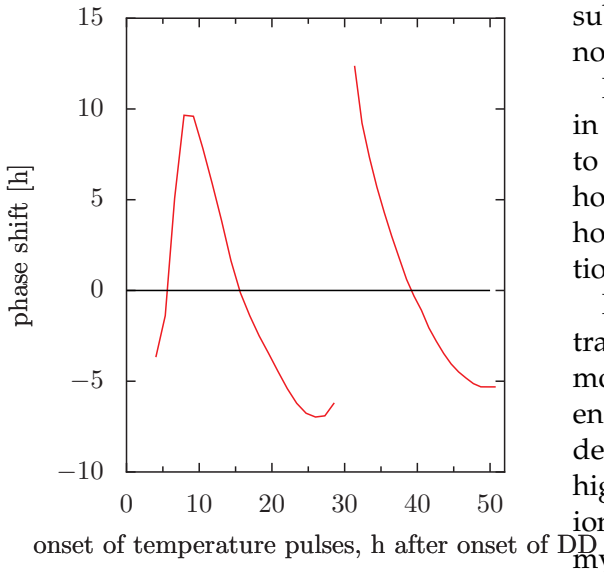

Figure 8.9: *Phase response curve of the petal movement rhythm of* Kalanchoe *to a 3 hour heat pulse of* 40^0C *administered at various times after onset of 'continuous darkness' (weak green light). Advances of the rhythm in respect to the control are plotted upward, delays downward (in hours). After [382]*

is reduced. Out of sucrose, glucose and lactose glucose has the least damping effect. For experiments using isolated flowers a concentration of 0.2 M glucose is optimal. The promoting effect of sugars on the oscillation is via the metabolism and not via a turgor effect, as shown in experiments using a lactose-analogue with the same turgor properties, but without metabolic effects ([392]). Turgor-affecting substances such as polyethylene glycol do not affect the circadian oscillator ([124]).

Heavy water (D_2O) slows the oscillation in a concentration dependent way from 23 to 26 hours (control versus 70% D_2O). 4 hour D_2O-pulses shift the rhythm up to 1.5 hours, depending on the phase of applications ([971]).

Ions such as K^+ and Na^+ up to a concentration of 0.5M have no effect on the petal movement rhythm ([1413]). Li^+ lengthens the period in a concentration dependent way. Concentrations of 5 mM and higher are toxic ([377]). As in *Albizzia*, ion carriers such as nonactin and valinomycin are without effect on the rhythm. If K^+ changes are responsible for the turgor changes affecting the circadian rhythm, ionophores should influence the rhythm heavily. And they do so in the case of *Lingulodinium* and *Phaseolus*. Perhaps the substances did not reach the motor tissue in the case of *Kalanchoe*? Or the temperature was to low: It is known that valinomycin effects are found only at higher temperatures.

Tetraethylammoniumchloride, a K^+-channel blocker, leads to phase delays of the petal movement rhythm if offered as a 3 hour pulse. Continuous application damps the rhythm, but the period is not affected ([329]).

Likewise, vanadate, a plasmalemma-ATPase inhibitor, delays the circadian

rhythm if given as a pulse. It has, however, no effect on period if applied continuously. Arrhythmic flowers are not re-induced to oscillate by a vanadate-pulse. Proton pumps are therefore no essential parts of the oscillator ([353]).

Alcohols influence the period length ([750]).

8.5.1 Effect of hormones

How plant hormones affect circadian rhythms has been reviewed by [822]. Abscissic acid is produced by plants under water stress and serves as a stress hormone. It was therefore tested whether it affects also the circadian clock. Single ABA-pulses phase shift the rhythm in a phase depending way, but period length is not affected (figure 8.10). This indicates, that the hormone does not influence the clock directly ([1337]). Jasmonate is a further hormone, which is produced under stress. In contrast to ABA it shortens the period of the petal movement rhythm, that is it affects the clock directly (figure 8.11). It is, however, not able to re-induce in arrhythmic flowers the oscillation. It therefore does not seem to interact with state variables, but only with the speed of the oscillator. Furthermore methyl jasmonate opens the flowers permanently, if offered for a longer time. It is known that methyl jasmonate induces swelling in potato tubers and other tubers. Perhaps methyl jasmonate is identical with metaplasin, a substance supposed to be responsible for the succulence of *Kalanchoe*-leaves in short day ([566]).

8.6 Models of the *Kalanchoe* flower clock

A feedback model of Johnsson and Karlsson was used to simulate the rhythmic petal movements of *Kalanchoe*. Originally this model was used to describe the gravitropic pendulum movement (see page 22). It can be applied also to a number of other rhythmic processes successfully. In the case of the *Kalanchoe* clock the phase shifting effects of light pulses and temperature pulses could be simulated quite well ([719]). Even the induction of arrhythmia by a special illumination was predictable (figure 8.12).

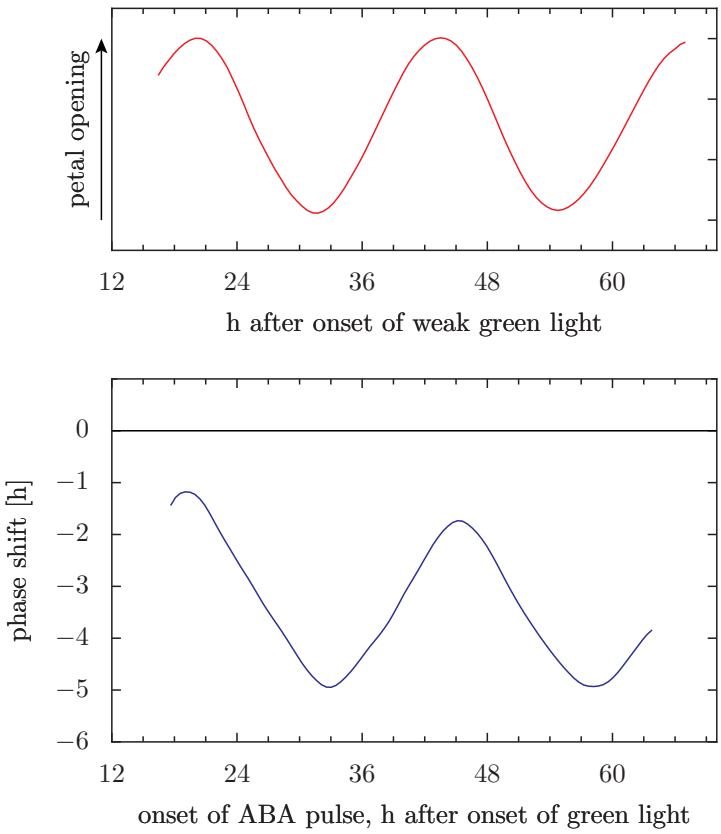

Figure 8.10: *Phase response curve by a 4 hour ABA-pulse* ($10^{-5}M$) *in the* Kalanchoe*-petal movement rhythm (below): Only delays of the rhythm in respect to the control were observed (negative values). The upper curve shows the petal movement rhythm of controls. Hours in weak green light after a 12:12 hour light dark cycle. After [1337]*

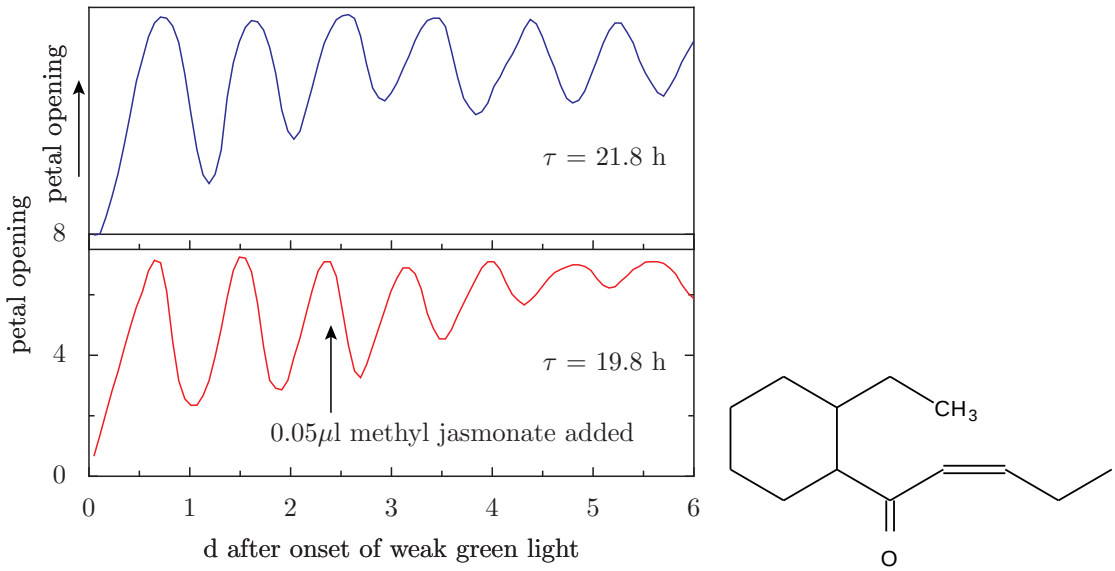

Figure 8.11: *0.05 µl methyl jasmonate (structure right below) was added at the time marked with a vertical arrow (lower curve). It shortens the period of the petal movement rhythm of* Kalanchoe *from 21.8 hours (control, upper curve) to 19.8 hours. After [391]*

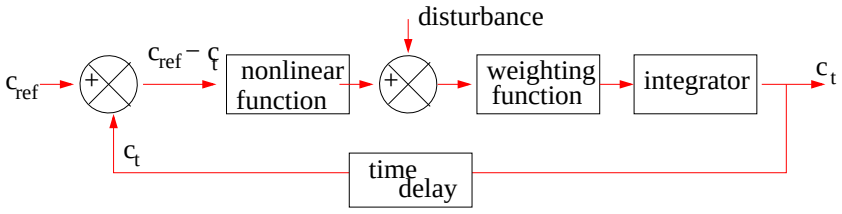

Figure 8.12: *A feedback model of Johnsson and Karlsson was used to simulate the* Kalanchoe *flower clock. A reference value c_{ref} is compared with a current value $c(t)$. If the two values differ from each other, the error signal $c_{ref} - c(t)$ is amplified by a non linear function f, weighted in a weighting function H (the preceding values influence the current value with varying weight), and after integration a new current value is compared again with the reference value. The size $c(t)$ fluctuates periodically, if the functions are supplied with the right parameters. After [717]*

9 Flowers and insects

Flowers serve the plants to reproduce and propagate. Flowers of many plants attract insects, birds or bats. These visitors pollinate the plants, which have developed numerous methods to prevent self fertilization and reinforce cross pollination. Nectar and pollen are offered and advertised by special colors, forms, fragrances of the flowers. Additionally, a coordinated timing with the pollinator is also important and widely found.
In this chapter rhythmic events are presented, which improve the interaction of insects and flowers. It will be shown that cross pollination is advantageous for plants. Many flowers open according to the species at certain times of the day or night, and insects such as bees use this time for visits. Different methods are used by the plants to attract insects. They emit for instance fragrances which attract especially night active insects. Often the emission of fragrance is under circadian control. Using the leaf cutter bee Megachile rotundata *as an example, the economic importance will be pointed out.*

9.1 Advantage of cross pollination

During the evolution of the organisms it turned out to be of advantage, to avoid self-pollination and use cross pollination instead. It increases the recombination of genes. This is the reason why so many mechanisms have evolved to prevent self-pollination.

This is the case also in flowering plants. Cross pollination (xenogamia) is widely spread among plants. Effective mechanisms to prevent self-pollination (autogamia) and neighbor-pollination (geitogamia) were developed. Besides the abiotic pollination (wind, water) the biotic pollination plays an important role: The pollen is transferred by animals. The plants offer food, protection and shelter. They advertise it by using colors, fragrances and special forms of the flowers. As in the advertisements of business men cheating is also used.

Very frequently insects are found as pollinators. They are attracted by the pollen and the nectar serving them as food. Flowers offer however sometimes also shelter and warmth. The flowers of some orchids use sex appeal as a method to attract males of certain insects. Finally flowers can serve as a breeding place. The symbiosis of the Yucca moth and the Yucca plant is an interesting example for it.

Flowers adapted during the course of evolution to certain groups of insects as pollinators. Special butterfly- and bee flowers exist. At the same time insects have adapted to the flowers. Perception, memorization and time sense are important in this connection. The next sections are concerned with these adaptations.

It is recommended to watch the movies of Baumann ('Lord of the flowers', 'Flowering marriage swindle' ('Blühender Heiratsschwindel') as a nice introduction. See also ([630], [327]).

9.2 Flower clock and time sense of bees

In 1747 Linné has constructed a flower clock. It shows, at what time certain flowers open and close. There are other rhythms found in flowers: Pollen- and nectar are offered, fragrances are emitted and heat produced in a rhythmic way. For the insects it is therefore of advantage, to remember the opening times of flowers in order to save energy and to start collecting at the most favorable time of the day or night. Furthermore it pays off to visit the flowers of the same species of plants. In this way they learn the peculiarities of the flowers and how to get the pollen and nectar in the fastest and most efficient way. Flower constancy in connection with a time sense are especially well developed in bees.

Bees can easily be trained to find food. This was observed by [445] while having his breakfast on the veranda of his vacation home. After some days bees arrived already at the table in search for food shortly *before* the jam was served. Once, when the family stayed inside the house because of bad weather, the bees still arrived and searched for food. Forell concluded, that the bees must have a kind of time sense which helped them to look for food.

Later von Frisch and his students did numerous training experiments with bees ([459]). The following signals are important for obtaining food: Fragrance, color, time and form of the flower (in this order). Fragrances are already successful with one training flight, colors need 3 to 4, training time 6 to 10 and form recognition 30 to 40. Mixtures of fragrances are characteristic for flowers and they can be distributed in the hive to the fellow forager bees. This is the reason why they play such an important role. The memorization of the fragrances disappears after some time, but reappears after 24 hours. This can be observed also under constant conditions. It is thus under circadian control.

Because pollen and nectar and the signals connected with it are offered only at certain times, bees use special collecting hours.

9.3 Daily opening of flowers

Quite a number of different plants open their flowers at different times of the day. They are pollinated and wilt after the seed and/or fruit has developed. *Pharbitis* is a good example: It opens its flowers in the morning (the English name 'morning glory' refers to it) and wilt in the evening ([1571]). Linné has proposed in 1751 a flower clock in which several plants are arranged in a circle according to their time of day or night at which they open or close (figure 9.1). From America the evening primrose *Oenothera biennis* (*Onagraceae*) has come to Europe which flowers from June to end of October. The developed flowers open in the evening between 20:00 and 22:00 o'clock very rapidly. It is fascinating to watch this event. Since each day new flowers have developed on the same plant, it can be observed during the course of several months. The rhythm is observable also under continuous light. It is thus endogenous ([24]).

Other plants open and close flowers during several days at certain times. The flaming Kat *Kalanchoe* is an example, which has been the topic of the last chapter.

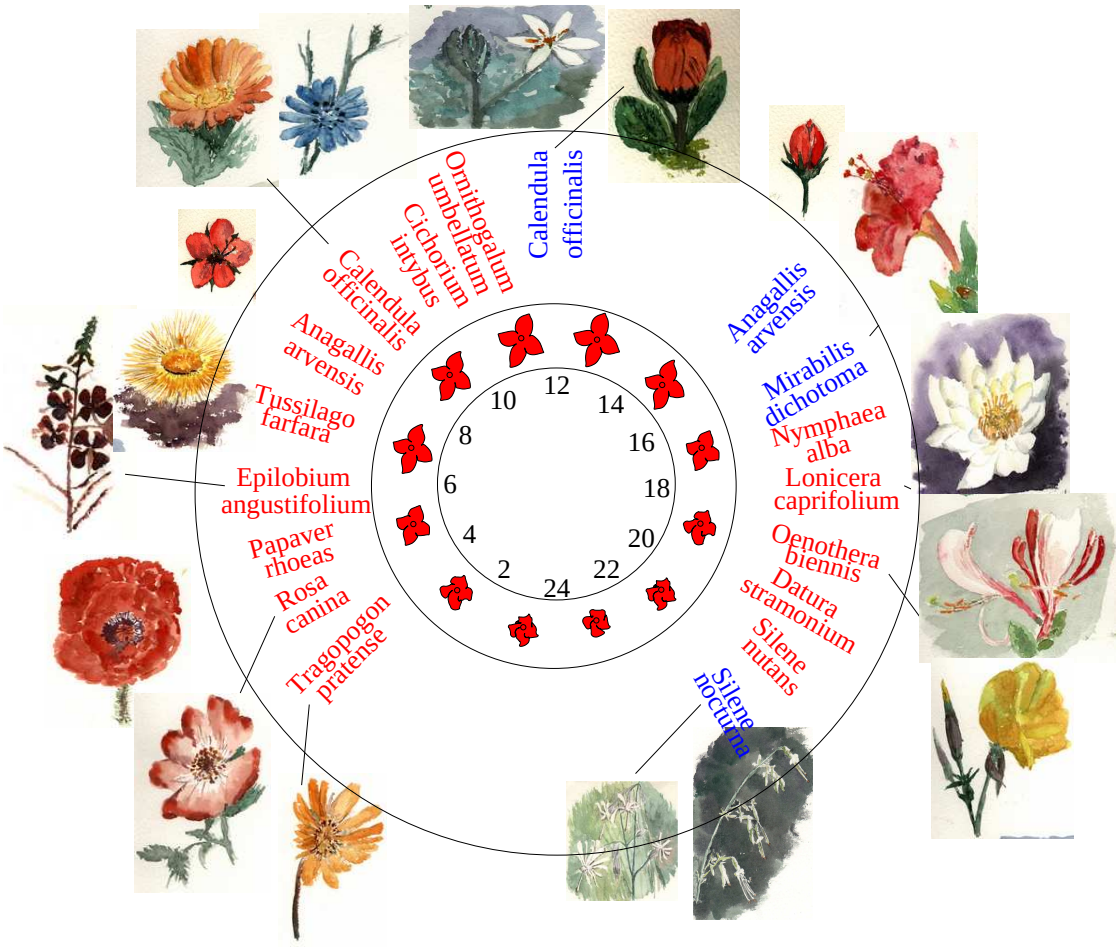

Figure 9.1: *Linné proposed a flower clock, which is shown in [122]. Here a flower clock composed by the author according to data from* [630]*, with added notes to the opening and closing times by Bünning, after* [723]

Figure 9.2: *Second day of flowering in* Parnassia palustris. *The first stamen has moved to the outer left. The second stamen pushed from behind over the closed pistil. Each day one of the five stamina moves to the periphery and a new one is positioned on top of the pistil. Flies visiting the flower are thus covered with the pollen and can pollinate other flowers of* Parnassia. *The pistil opens not before all the anthers are wilted and discharged and can now be pollinated with pollen from other flowers*

9.4 Other examples for securing pollination

In *Parnasssia palustris* each day one of the five stamina is pushed to the tip of the carpels. The stamina opens at the top and the pollen can be distributed by flies. On the next day this stamen moves to the periphery, wilts and is discarded. A new anther is shifted to the top. The pistil does not open until all stamina are gone. In this way self pollination is prevented ([630], figure 9.2). Pollen is offered by some plants to the pollinators at certain times of the day only ([183]).

In other plants the production and composition of the nectar fluctuates diurnally ([1153]). If the nectar secretion is limited to certain times of the day, closely related species can be phenologically isolated. In the jungles of Trinidad *Anguria*, a pumpkin plant, is pollinated by the butterfly *Heliconius*. Nectar secretion of *A. umbrosa* occurs between 7 and 12 o'clock, that of *Anguria triphylla* from 12 to 19 o'clock (figure 9.3). Fragrances are often emitted at certain times of the day only. The pollinators of these plants, insects, birds and bats, use their own clocks and orientation mechanisms, in order to adapt to the diurnal pattern of the plants.

9.5 Fragrance rhythms

How the intensity of fragrances of flowers changes during the course of the day has so far not very intensively been studied. In several cases it has been shown that fragrance emission is controlled by an endogenous rhythm. The flowers of the Night blooming Jasmine *Cestrum nocturnum* for instance are fragrant during the night and without fragrance during the day ([1122]). It was shown in the 'headspace' using gas chromatographic methods, how the intensity of the fragrance of the flowers of this plant and that of *Hoya carnosa* changes during the day ([967],[11], [12]). Whereas in *Hoya carnosa* the fragrant compounds are emitted synchronously, in *Stephanotis floribunda* the maxima of the fragrant substances 1-nitro-2-phenylethan and methyl benzoate are shifted in respect to each other by 12 hours ([967] and figure 9.4). In this way the composition of the fragrance changes drastically. Some tobacco species are pollinated mainly by night active insects. They are very fragrant during the night ([924]). The day fragrant plants (*Citrus aurantium*, *Odontoglossum constrictum*) studied so far do not show an en-

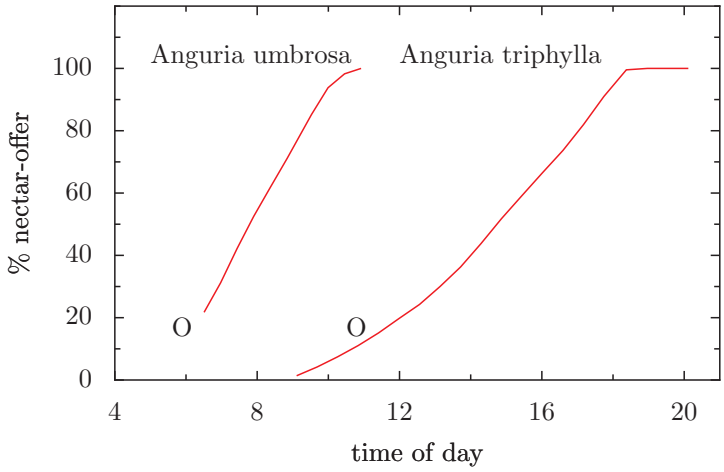

Figure 9.3: *The nectar secretion of* Anguria umbrosa *occurs between 7 and 12 o'clock (green curve), the pollen presentation briefly before it. In* Anguria triphylla *(red curve) the nectar is presented between 12 and 19 o'clock, the pollen between 10 and 12 o'clock. After [630]*

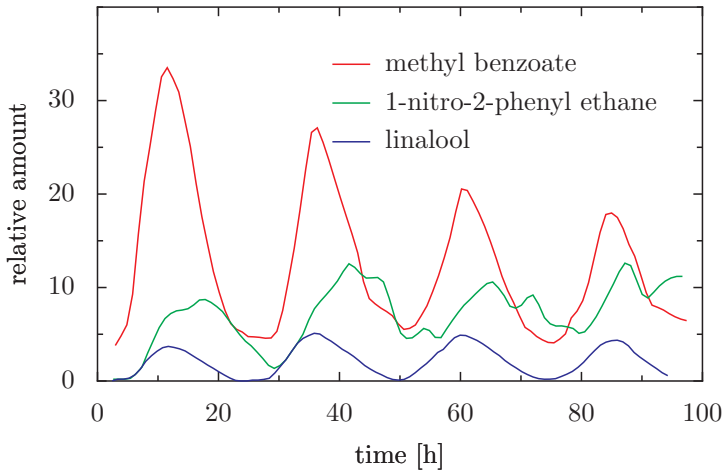

Figure 9.4: *The fragrance production of* Stephanotis floribunda *was measured in the headspace. The compounds methyl benzoate and linalool are emitted around the same time of the day, although in varying amounts. The 1-nitro-2-phenylethan emission rhythm, however, is shifted against the rhythm of the two other fragrant substances by 12 hours. After [967]*

dogenous rhythm in emitting fragrances. Examples for fragrance rhythms are presented under the special topics in chapter 20. Substances are also used by higher plants as SOS-signals as a means of protection from being attacked by animals: These fragrant substances attract natural enemies of the attacking insects, such as parasitic wasps. Parasitic wasps are in addition attracted also by the smell of the excrements of caterpillars ('kairomones', [1491]).

9.5.1 Fragrant substances of flowers

At least 30% of all higher plants produce volatile substances. In higher concentrations they are toxic to the plants. Therefore they are deposited in special cells of the surface of flowers, leaves, stalks or even roots as etheric oils. In some plants the fragrances are collected in special fragrance glands (osmophors). These and other fragrance spots lead the pollinators in combination with colored nectaries to the parts of the flower which are important for pollination. Insect flowers use colors and fragrances which differ from the one used by bird- and bat-flowers. Fragrances and fragrance spots affect the pollinators during the day at short distances and facilitate the orientation in the interior of the flower (osmotaxis). Night flowering species on the other hand possess an intensive fragrance, which intensifies with the onset of darkness. Night active moths such as *Sphingidae* are stimulated to land by fragrant substances ([150], [151]). Furthermore, the flowers of night flowering plants are white. This color is more easily found by flying insects.

Fragrances of flowers are usually composed of many substances. The most frequently found compounds are isoprenoids (=terpenoids) and benzenoids (figure 9.5). Furthermore aliphatic compounds and substances with heteroatoms are found. Nitrogen containing compounds such as indole or methylanthranilate can influence the fragrances of flowers heavily. Sulfur containing compounds are also found in fragrances of some flowers.

Fragrant substances are used in the perfume industry. They are extracted with solvents or water vapor. Many fragrant compounds are nowadays synthesized.

9.6 Economic significance of pollination: Leaf cutter bee *Megachile*

The pollination of flowers by insects is of economic importance. Many cultivated plants such as orchard trees have to rely on it.

The significance of insects for the pollination of plants can be illustrated by the example of the lucerne ([330]). Lucerne is especially in the United States an important food crop. To obtain seeds large lucerne fields have to be pollinated. Bees are of no use for it. They are deterred by a special 'trigger mechanism' of the lucerne flowers. In 1930 the leaf cutter bee *Megachile rotundata* (*Hymenoptera*: *Apoidea*) was introduced to the United States (figure 9.6). It originates from Eastern Europe and western Asia. It lives solitary and pollinates lucerne very effectively. The insect uses furthermore pieces of the leaves of lucerne, in order to surround the breeding cells in hollow stalks and empty snail shells. An egg is laid on top of a pollen-nectar mixture and covered with a piece of leaf. Further breeding cells are produced

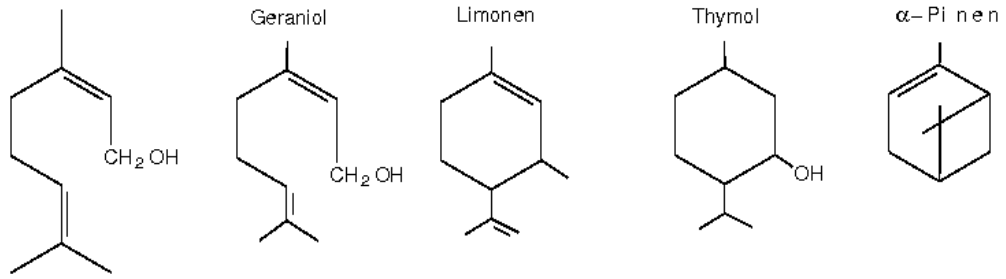

Figure 9.5: *Chemical formula of some fragrant substances: Geraniol, limonen, thymole and α-pinen. From [630]*

and an egg added to the food until all the available space is used up. With a piece of leaf the whole art work is sealed off. To pollinate one hectare of land 5000 females are needed. A whole industry has developed in the meantime, which rears and sells *Megachile*. It is profitable for the farmer to buy these bees, since he gets 22 dt[1] lucerne seed instead of 1-3.5 dt per hectare without these bees.

Figure 9.6: *Leaf cutter bee* Megachile rotundata, *an important pollinator of lucerne-flowers. From the author after [330]*

Megachile hibernates as a prepupa. This diapause is not induced by the day-length, as is the case in most other diapause examples (see page 259), but by thermoperiodism: The day-length is measured by the higher temperature during the day ([1493]). Diapause occurs in a tightly spun cocoon in the prepupal stage (La3). The cocoon is surrounded by cut pieces of leaves (figure 9.7) and is placed in small dark cavities or in a hole. Day-length does not induce diapause even in animals placed in a gelatin capsule. At 7^0C diapause can last up to two years. It is terminated by temperatures above 17^0C ([1419]). The larva chews its way through the cell. The eclosion rhythm is also insensitive against the light-dark cycle. A respi-

[1] decitons; 1 dt = 100kg

Figure 9.7: *Megachile rotundata* in cells which are produced from pieces of cut leaves by the mother and filled with pollen before the egg is deposited. The larvae develop in larval stage three to a prepupa. A dense cocoon is spun, in which diapause occurs. Induction of diapause is under thermoperiodic control. Two of the cells are opened and show the animals inside

ration rhythm was recorded, which is ultradian during the larval stage and circadian in the adult. The respiration rhythm does not react to light cycles either, but does so toward temperature pulses. A phase response curve toward temperature pulses was obtained. More on thermoperiodism in [1313].

10 Sun compass orientation

Several insects and other animals use the direction of the sun for orientation. Bees are able to inform others on the direction and distance of food sources from the bee hive. The crustacean amphipod Talitrus *uses the sun compass for its orientation at the beach of the sea. In another amphipod,* Talorchestia, *which lives in areas close to the equator, this compass works also if the sun passes the sky in the north instead of the south. Other examples of sun compass orientation are mentioned.*

If insects search for pollen and nectar, they must perceive signals of the plants. They are, however, also able to use earlier experience or even -in the case of bees- the experience of other worker bees. To save time and energy for collecting food, sun compass orientation is used.

Sun compass orientation was discovered by [1307] and [175] when studying ant behavior. Thereafter is was found and studied also in bees ([458]) and birds ([827]), bird migration see [99]). Later this ability to orientate was observed in many more groups of animals. A recommendable overview is [1331]. We will have a look on two examples, the sun compass orientation of bees and of beach hoppers.

10.1 Sun compass orientation and communication in bees

Bees use the sun for orientation while looking for food. They are able to transfer the information on food sources to other forager bees (round- and wiggle dance), convey not only the kind of food (fragrance, pollen) and the quality and amount, but also the direction and distance. Other forager bees find in this way food faster and they need less energy. To convey the direction, the angle of the food source to the sun is coded in a wiggle dance. Since the bee hive is dark, the direction to the sun is transformed vertical up and the angle to the sun is transferred to the fellow foraging bees by the direction of the wiggle run (figure 10.1). This sun compass orientation functions even under overcast sky with small patches of open sky. The foraging bee uses in this case the polarization pattern of the sky and is able to extract the sun direction from it. If individual foraging bees are caught on there way from the food source back to the bee hive and kept for some time in the dark, they can still convey the direction of the food source correctly to other foraging bees. Since they were not able to observe the path of the sun during their captivity, they must possess an endogenous clock with a 24-hour rhythmicity telling them the passed time. Other rhythmic events in the bees life are controlled by this clock such as the memorization of fragrances and food sources after 24 hours.

Thus the sun compass orientation helps the bees to orientate, to collect food, and to communicate.

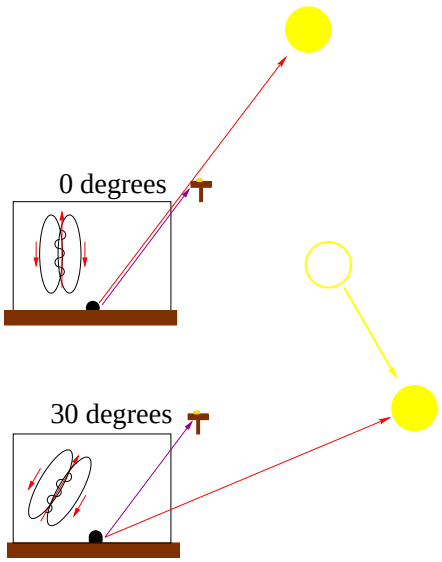

Figure 10.1: *Sun compass orientation of the honey bee and wiggle dance. In the upper part of the figure food is located in the direction of the sun. The foraging bee projects the sun to the upward plumb line in the vertical comb of the bee hive. In this way it conveys the direction of the food to its fellow forager by performing the illustrated 'wiggle dance'. They walk down at the side, than in the center upward by wiggling the abdomen (the frequency of the wiggling stands for the distance of the food source), and than at the other side downward again. In the lower part of the figure the food is located 30° left of the sun. The wiggle dance is correspondingly indented by 30° to the left. After [459]*

10.2 Sun compass orientation of the beach hopper

At the coasts there are zones with different physical and biological properties ('ecotonal system'). These zones are periodically shifted due to the tides and aperiodically by storms. In these zones a special fauna is found. It must adapt constantly to the changing conditions of life. Some organisms stay in specific zones or try to reach them. Others, however, migrate through these zones and show thereby different behaviors.

The beach hopper *Talitrus saltator* Montagu belongs to the amphipods (*Malacostracae*), an order of the crustaceans (figure 10.2). It is frequently found in the wet sand of European coasts (figure 10.3). It lives at the beach close to the high tide line. During the day it is sheltered in the (not too) wet sand. During the night it undergoes excursions up to 100 meters toward the inland. If during the day it becomes too dry, it returns in the direction to the sea. It does not need to see the sea, but can make use of its build in sun compass. This was shown by experiments using mirrors to change artificially the direction of the sun and by using an artificial sun (light source) ([1137]). Only the azimuth and not the heights of the sun serves for orientation. Differences in the azimuth (geographical latitude, season-, northern and southern hemisphere) are taken care of. In contrast to ants these differences must not be learned ([693]).

The escape direction of the population depends on the position of the coast. It is genetically programmed for the particular population. If animals with different flight directions are crossed with each other, the hybrids use an intermediate flight direc-

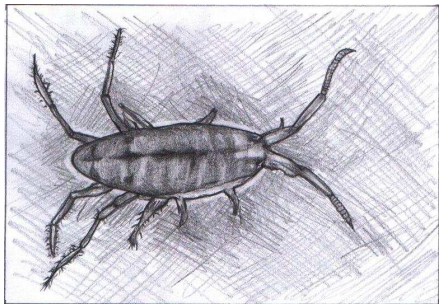

Figure 10.2: *Beach hopper* Talitrus saltator *(Amphipods), a crustacean. Drawn from Mareike Förster after [1138]*

tion. Instead of the sun the pattern of the polarized light of the sky can be used for orientation. Further aids for orientation are the slope of the beach, land marks, a magnet compass. Without these additional aids the astronomic direction finding would be less precise.

During the night the moon is used for orientation ([1136], [1135]). This works even after several days of darkness before the test is performed ([1135]). To measure the time an oscillator is used with a period length of 24 hours and 50 minutes and not an hour glass method ([396]).

With a simple experiment ([1138]) it was shown that the animals use the sun for orientation. For this, beach hoppers were captured and positioned in the center of a glass vial. This vial allowed the animals to see the sun, but not the sea and the land. Under these conditions they jump in their flight direction. If the sun is blocked by using a cardboard as a blind and the sun is reflected with a mirror from another direction onto the animals, they escape as if the mirror sun is the real one (figure 10.4). These beach hoppers can find out the current angle to the sun and use this information for their flight direction. If the sun *is* used by the animals for orientation, they

Figure 10.3: *Beach biotope of the beach hopper* Talitrus saltator. *Drawn from Mareike Förster after [1138]*

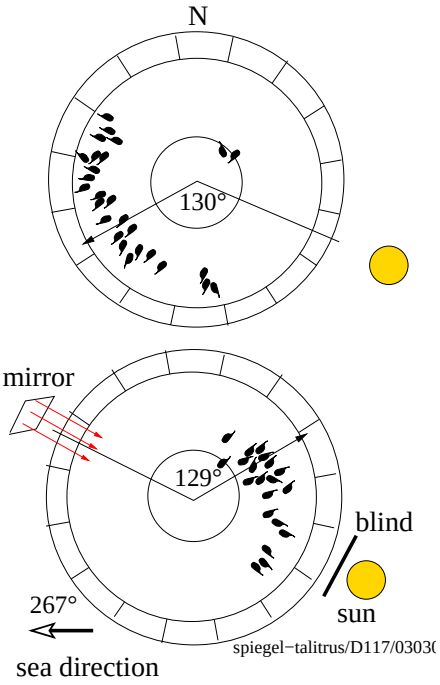

Figure 10.4: *Sun compass orientation of the beach hopper* Talitrus saltator. *It uses the sun for orientation if its local beach place is getting too dry (top). If the sun is reflected with a mirror,* Talitrus *uses the mirror-sun (below). After [1138]*

must change the angle slowly, since the sun is traveling from east to west. And this is indeed what they do: The path of the sun is taken care of. That is, they must possess a clock which is used as a time reference. If the light-dark-cycle of the animals is shifted by artificial illumination which does not correspond to the natural day, their time reference is also shifted. The orientation of the animals shifts correspondingly (figure 10.5).

10.2.1 Sun- and moon orientation at the equator

How *Talorchestia martensii* orientates at the equator, was studied at the Indian ocean

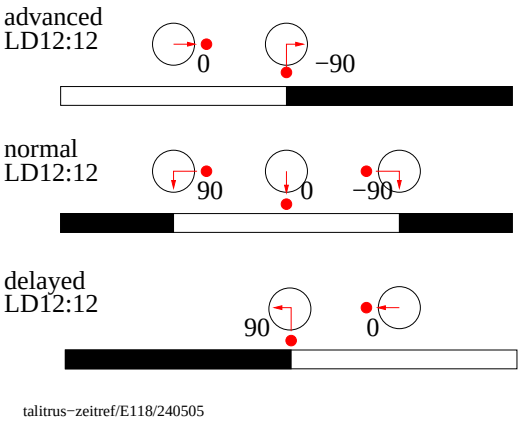

Figure 10.5: *If the light-dark-cycle of the beach hopper* Talitrus *is shifted by an artificial illumination which does not coincide with the natural day (6:00 onset of light, 18:00 end of light), the time reference of the animals is shifted and their orientation changes correspondingly: A light-dark-cycle which was phase advanced (top) changes the synchronization of the circadian clock of the animals in such a way, that they now escape in the morning at 6 o'clock (their noon) in sun direction and at 12 o'clock in a way controls would do during their evening (center) (and it is their subjective evening). A light-dark-cycle which is by 6 hours delayed (bottom) changes the synchronization of the circadian clock of the animals in such a way that they now at 12 o'clock (their morning) escape toward east instead south. At 18 o'clock they escape in a way the controls would do during noon (center) (and it is their subjective noon). Thus the animals use their internal clock for orientation. After [1138]*

in Somalia and Kenya. The animals live between the upper supralitoral and the lower eulitoral. They are adapted to the local tides with a tidal and a diurnal component. In latitudes close to the equator the sun passes over the sky either north or south, depending on the time of the year. Therefore the differences in azimuth are strong. In spite of this the animals are able to use the sun compass. They orientate additionally with the help of the magnetic field of the earth ([1138]).

10.3 Further examples of sun compass orientation

In ants ([1307], [175], a newer account is [1537]) and spiders ([1134]) sun compass orientation was established. The spider *Arctosa cinerea* lives at the banks of European rivers from Finland to the Mediterranean countries. Italian populations were unable to orientate themselves during the summer in Scandinavia at sun times which where unknown to them. The local Scandinavian populations, however, orientate themselves also during the midnight sun.

Locusts belong to the grasshoppers. There are ten typical locust species. *Locusta migratoria* is the most common. The swarms can migrate for several thousand kilometers. In doing so they follow the wind, but orientate also according to the sun and moon.

Among the butterflies the monarch (*Danaus plexippus*) migrates during the late summer and fall covering distances up to 3000 km. Here again a sun compass is used.

11 Clocks which run according to the moon

After explaining briefly how tides are brought about and how this affects the coastal biotops, examples for tidal rhythms in the isopod Excirolana, *fortnight rhythms in a terrestrial crab and the eclosion of* Clunio *are presented. Finally, monthly rhythms and their significance for the organisms at the oceans are explained.*

While orbiting once in a year the sun, the earth spins once during 24 hours around its own axis. Its satellite, the moon, takes, however, 24.8 hours for a turn. Therefore the constellation earth-moon-sun changes continuously, but in a regular order. According to Newtons law of gravity ($k = m_1 * m_2/d^2$, where k is gravitational attraction, m_1 mass of body 1, m_2 mass of body 2, d distance between the two bodies) two celestial bodies such as earth and moon attract each other. The attracting force decreases with the square of the distance between the two bodies. This is shown in figure 11.1 for the point A on the earth surface facing the moon, the center of the earth M, and the point B on the earth surface opposite to the moon by the different sizes of the arrows.

Each day there are two tides of the oceans observed. How do they arise? The gravitational force of the moon could be responsible for attracting the water masses of the part of the earth facing the moon. Earth and moon turn around their common center of mass once in a sideric month (27d 7h 43 min). Since this center of mass is not the center of the earth, but is about

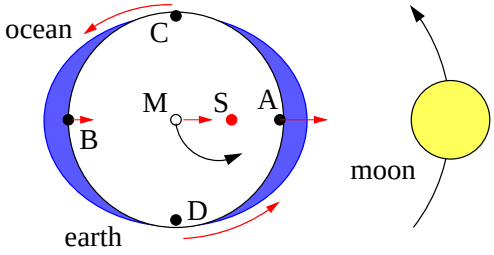

Figure 11.1: *Gravitational acceleration (red arrows) of the moon (yellow) on different points on earth. In A it is stronger as compared to M, the center of the earth (x), and there it is stronger as compared to point B at the opposite side. The center of the globe M turns around S (red point), which is the center of mass of the system earth-moon. The waters of the oceans (blue) are drawn away from C and D by the differences in gravitational acceleration of the moon. The result are* two *tides per day (12.4 hours apart). Note that the waters are not lifted up (the gravitational forces are much too small), but tangentially withdrawn from C and D toward A and B. After [751]*

2/3 away from it (red point S in figure 11.1), centrifugal forces develop. They could be responsible for the second tide on the opposite side of the earth. However, these explanations are wrong. First of all, the gravitational acceleration, which binds the water to our planet, is 300 000 times stronger as the one of the moon. The moon is therefore not able to lift the water masses of the oceans up. Secondly, the tides at the opposite side to the moon are almost as high (just 4% less) as the tides on the surface of the earth facing the moon. Thirdly, the centrifugal forces of the earth-moon system are very low and can not explain the bulging of the water masses on earth at the opposite side of the moon.

The correct explanation of the cause of the tides is the following (see figure 11.1): In point A the water bulges, because the moon accelerates the water more strongly as the earth does. Since our planet rotates faster than the moon, the water masses are *tangentially* torn away from the solid earth. In point B the water stays back due to its mass inertia, while the solid globe is torn away from the water. In this way the waters are moved away from the points C and D toward A and B. The moon does not lift up the water, but moves it tangentially along the earth´s surface. The period between two high tides is 12 hours and 25 minutes.

In addition, the tides are also influenced by the sun. The sun is 400 times more distant as is the moon, but has a 1800 times larger gravitational acceleration. However, since the gravitational acceleration is proportional to the reciproce of the 3rd power of the distance l ($b = 2Gr * m/l^3$, where b is the gravitational acceleration, G the gravity constant, r the earth radius, and l the distance earth-sun), the gravitational acceleration amounts to 45% of that of the moon only. During syzygia (full moon, new moon) the forces of the moon and sun add up and lead to spring tides, whereas during half moon the forces of the sun reduce those of the moon and neap tides result (figure 11.2).

The tides on earth are further complicated by the elliptic orbit of the moon around the earth. During perigeum the moon is 9 to 14 % closer to the earth as compared to an apogeum. The tidal effects are therefore 30 to 48 % stronger. In combination with sycygia extreme tides (‘perigean spring tides´) arise.

Other factors influence the tides. The flood bulge is located at the sublunar point (the place on the earth surface where the moon passes the zenit); this place depends on the moon declination.

Quite a number of rhythms affect the tides: Half of the lunar day (12h25min), half of the sun day (12 hours), half of the synodic month (14.77d), half of the sideric month (13.66d), the anomalistic month (27.55d), half annual variation of the sun declination (182.6 d), the anomalistic year (365.26d), the prograde year (8.8years), retrograde turn of the nodeline (18.6y).

Tidal effects are mainly found at the coasts of the oceans. Geophysical factors such as resonance properties of the ocean, current straits, course of the coasts, local features such as funnel shaped river mouths influence the tidal pattern and the height of tides. Due to these factors and their various combinations the tidal lift, which is 35 cm only at the open sea, can accumulate at the coasts and reach heights up to 4 m (German North Sea), 7 m (French Atlantic coast) and 21 m (certain funnel shaped river mouths).

The kind of tidal movements can be quite different: Usually there are tides with two low tides and two high tides,

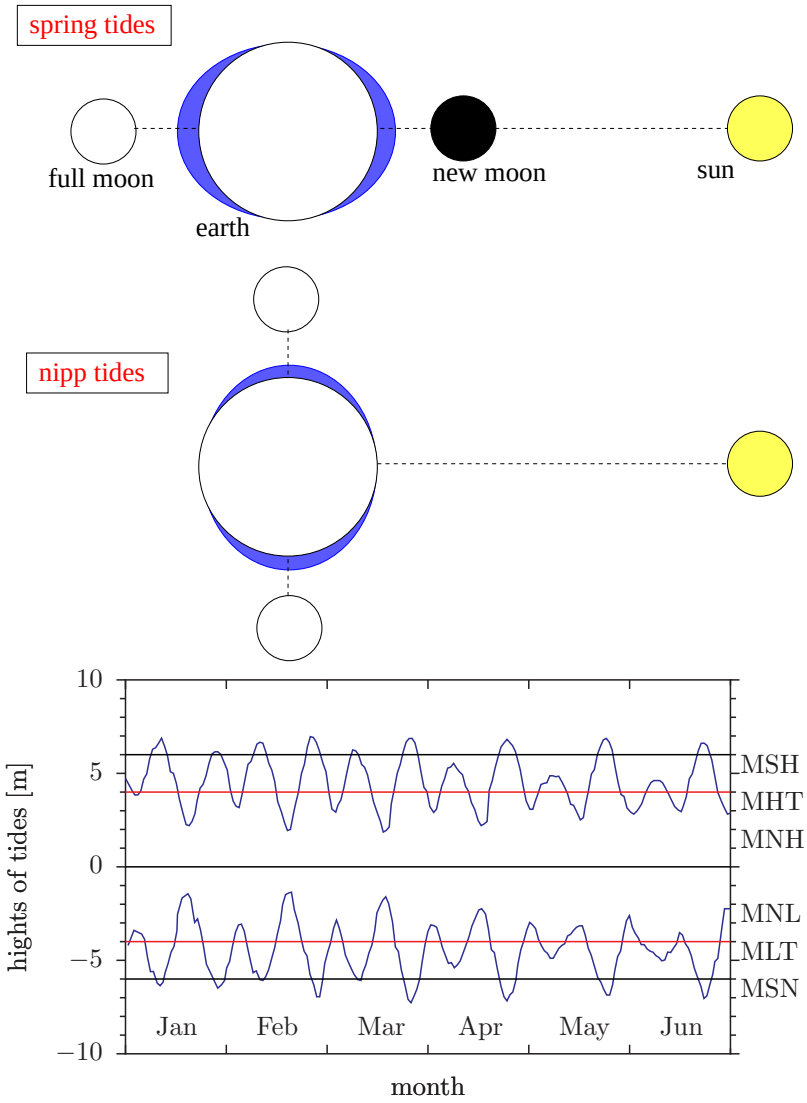

Figure 11.2: *Monthly occurrence of spring-and neaptides. If moon and sun are lined up with the earth (new moon or full moon, upper part), the tides are enforced* (springtide)*. If moon and sun are perpendicular to the earth (first and last quarter of the moon), the heights of the tides are reduced* (neaptides)*. As a result the tides are modulated in amplitude during the course of a month (bottom, MHT: mean high-tide, MLT: mean low-tide, MSH: mean spring high-tide, MSN: mean spring neaptide, MNH: mean neap high-tides, MNN: mean neap low-tides, in meters, ordinate). After [1129]*

but mixed forms and tides with only one change of high and low tide per day are found. For an overview see [60].

In the eulitoral zone (between the highest high tide and the lowest low tide) the conditions change drastically (figure 11.3). Depending on whether this zone is exposed to the surf or protected from it, whether the coast is flat or steep, temperature, humidity, flooding, oxygen content and food supply, salt content, pressure, undulation and light conditions change. See [1083] for an overview. The differences in the tides can be just a few centimeters or amount to more than ten meters. If the coast is very flat, the tidal zone might cover several kilometers.

The organisms living at the coasts and oceans have to adapt to these tides. We find therefore tidal rhythms, fourteen day and 28 day rhythms in these organisms. In the following some examples for tidal, fourteen day and monthly rhythms are presented. For literature see [1074], [1129], [147], [1131].

11.1 Tidal rhythms

Tidal rhythms are widespread among crabs (e.g. fiddler crabs), crayfish (*Carcinus*, *Emerita*, locomotor activity and color change of the carapax), mites at the sea shore, and in shells (limpet *Patella*). Only a few insects with tidal rhythms have been described. A terrestrial beetle, *Thalasotrechus barbarae*, is an example. A cave cricket (*Ceuthophilaus maculatos*) was claimed to possess a tidal rhythm. In fish, *Blennius* show tidal rhythms ([495], [496], [497]) and birds such as the reef-heron, which flies with a tidal pattern to the sea. Even in unicellular algae tidal rhythms have been reported such as the

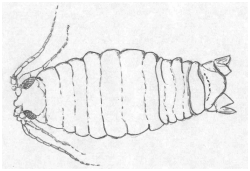

Figure 11.4: Excirolana chiltoni *(isopode) from the coast of California*

vertical migration of the commuter diatom *Hantzschia virgata* ([1130]) and of euglenoid algae ([1132]). The astonishing precision of the activity pattern of *Excirolana chiltoni*, an isopode of the Californian coast (11.4) in respect to the tidal pattern of the ocean is shown in figure 11.5 and 11.6. During low tide the isopode is buried in the sand, during high tide it swims for about two hours about to search for food. The intensity of the activity depends on the height of the high tide. The swimming behavior can be simulated in Petri dishes with sand and sea water in the laboratory. Even in this situation the mixed semi-diurnal tidal patterns of the Californian coast is exhibited by the animals. This is not the direct consequence of Zeitgeber, since the activity rhythm recorded in the laboratory steps slowly out of phase with the natural conditions.

Time cues for the synchronization of this endogenous rhythm in the natural biotope might be pressure differences by periodic inundation, change in water and thus chemical concentration differences such as salt content, temperature differences or water turbulence. The light-dark change has of course no synchronizing effect in tidal rhythms. Out of these possible Zeitgeber it turned out that in *Excirolana* water turbulence is effective. They can be simulated by shaking the vials with a wave sim-

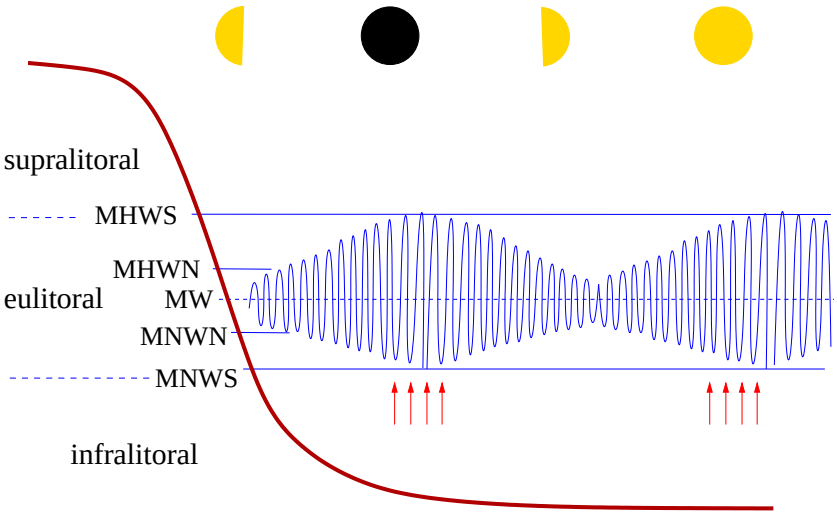

Figure 11.3: *Supra-, eu- and infralitoral at the sea coast with mean high tide of the spring- (MHWS, blue line) and of the neap tides (MHWN) and mean low tide of the neap- (MNWN) and spring tides (MNWS). Mean water level MW stippled blue line. Neap tides after half moon (top), spring tides after full- and new moon. The red arrows at low tide during the spring tide period of the water movements refer to the eclosion days of the* Clunio *midge (page 192). After [211] and [1072]*

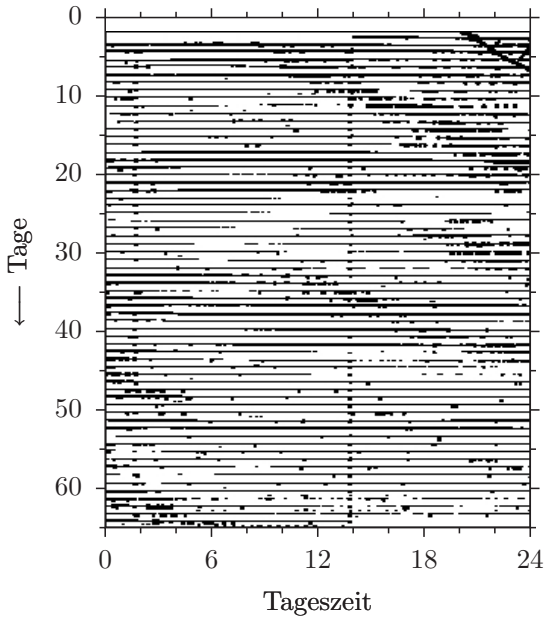

Figure 11.5: *Activity pattern of the swimming behavior of a beach hopper* Excirolana chiltoni, *a 'virtuoso' isopode from the Californian coast. The actogram represents the daily activity of an animal (consecutive days 1 to 65 below each other). After [396]*

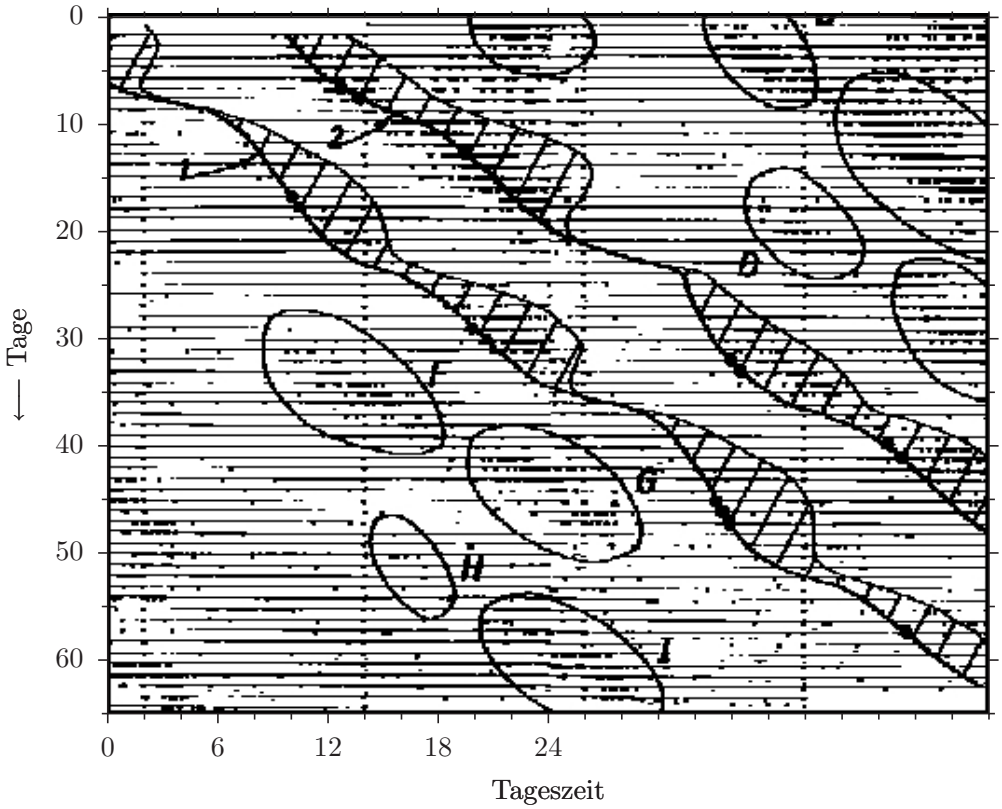

Figure 11.6: *The activity pattern of the swimming behavior of* Excirolana *from figure 11.5 has been duplicated, so that day 1 and day 2 are in one line, day 2 and day 3, day 3 and day 4 and so on. Line 1 connects one of the daily spring tides and line 2 the second daily spring tide. The small dots mark the days with maximal spring tide. The obliquely marked areas represent for each day the tide heights. The circled parts A to I of the actogram correspond to increased activities and correlate well with the tidal patterns as indicated by the lines 1 and 2 and the hedged areas. After [396]*

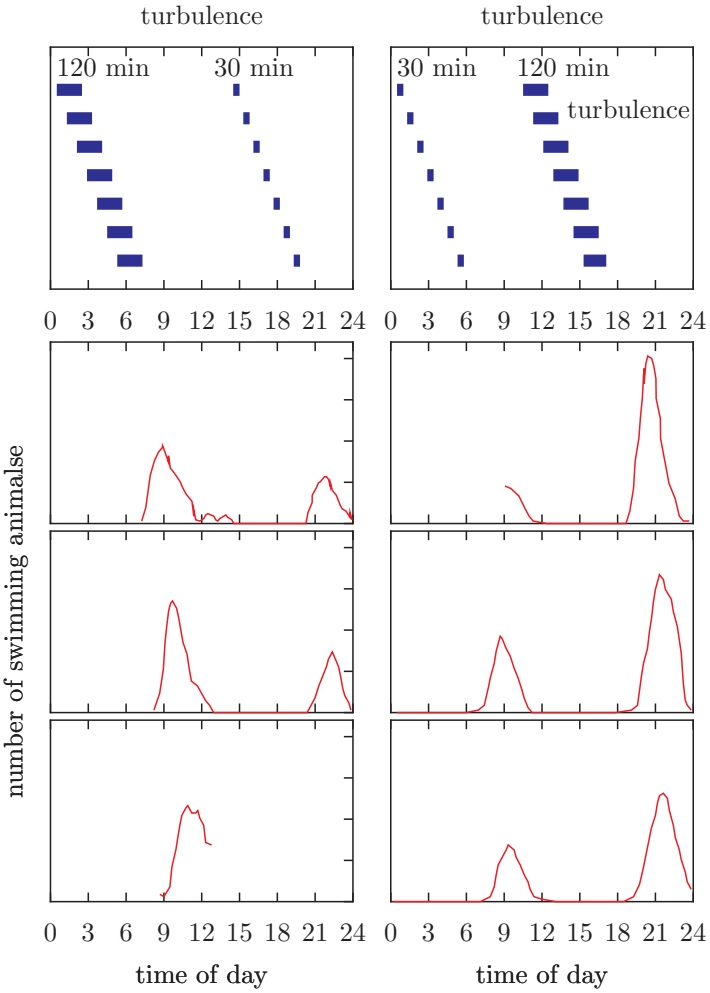

Figure 11.7: *In* Excirolana chiltoni *a long shaking period (120 minutes) was followed 6 hours later by a short shaking period (30 minutes) (left part of figure). The swimming activity of the animal was measured afterward under constant conditions without shaking periods. The swimming pattern in the lower part left with high activities and 6 hours later with less high activities was obtained. If, however, first a short and afterward a long shaking period was administered (right upper part of figure), a period of less high activities is followed 6 hours later by a period of high activities (right lower part of figure). The pattern of the tidal rhythm is thus reflected by the activity pattern. After [782]*

ulator (shaker, magnetic stirrer, [782]). The length of the shaking period determines the form of the rhythm: If a long shaking period and a short one are presented with tidal distances, the longer one induces a stronger activity push as the shorter (figure 11.7).

A phase response curve to shaking pulses at different times of the day under constant conditions was established by Enright.

Delays occur immediately, advances after more than 2 transient cycles. The bimodality of the actograms is amplified, if the shaking occurs in the middle of the two activity periods.

The curve shows two peaks per day (Enright in [302], see figure 11.8). Enright interprets the bimodal curve as a bimodal circadian rhythm synchronized by the tides. Other researchers assume, that a tidal rhythm is responsible for the two peaks per day, and some of them believe that we are dealing with circalunidian rhythms (discussed on page 112 of [1131]).

Alcohol and heavy water D_2O increase the period length of the tidal rhythm ([395], [394]).

Temperature compensation of tidal rhythms was established in *Excirolana chiltoni*, *Clunio* ([1076]) and *Carcinus maenas* ([1062]).

Tidal rhythms are not only found at the shores of the oceans, but can be measured also on the mainland. [1621] found a time course parallel to the gravimetric tides in the inland (Switzerland) in the diameter of logs of wood (figure 11.9).

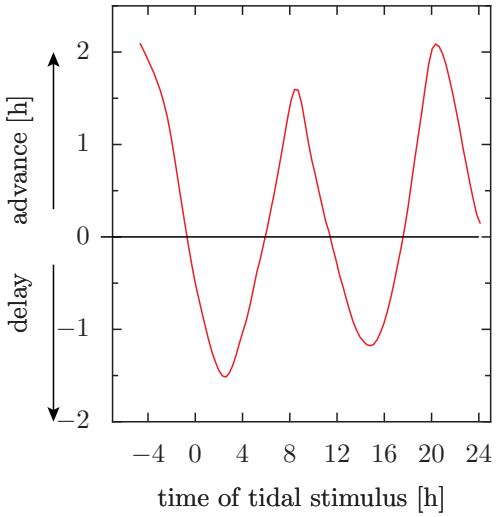

Figure 11.8: *The swimming activity of* Excirolana chiltoni *was measured in individual animals or groups for three to four days under free run conditions. Thereafter the animals or groups were exposed at different phases of the cycle (abscissa) to a tidal stimulus in form of shaking (every minute for 10 seconds) for two hours. Afterward the swimming activity was recorded again under constant conditions. The shift of the rhythm in respect to the rhythm before the stimulus was plotted as advance (y-axis upward) or delay (y-axis downward) in a phase response curve. After Enright in [302]*

11.2 Fortnight rhythms

Two examples are given, the liberation of the larvae of the terrestrial crab *Sesarma haematocheir* and the eclosion of the one hour midge *Clunio marinus*. Two movies are available for fourteen day rhythms: One on the grunion *Leuresthes tennis* ([1525]) and one on the one-hour-midge *Clunio* ([1071]).

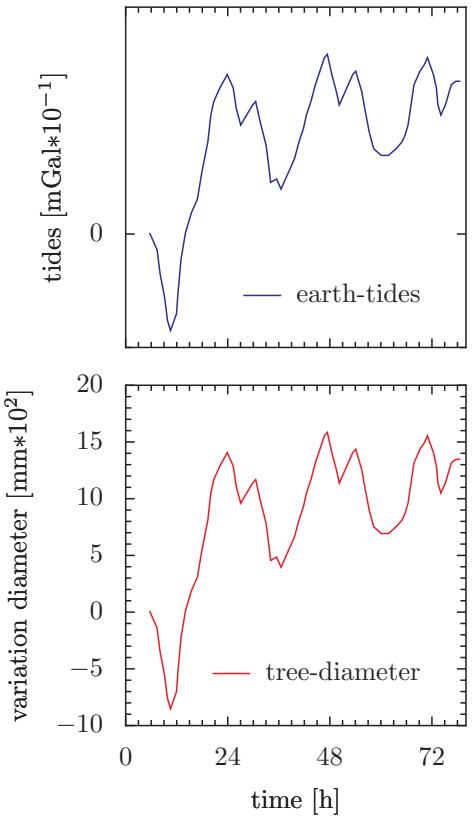

Figure 11.9: *The diameter of a log of wood changes during the course of days (x-axis) parallel to the gravimetric tides in the inland (Switzerland). After [1621]*

11.2.1 Fortnight rhythm of the terrestrial crab *Sesarma haematocheir*

Sesarma haematocheir is a terrestrial crab which is common in Japan. Different populations live in quite divers habitats. But all of them must take care, that the larvae are transported to the sea. One of the populations of this species live as adults in the mountains above the Ogamo river close to Kyoto. The crabs copulate during the summer. The zygotes are discharged and stick to hairs at the ventral side of the abdomen of the females. After the larvae have reached the zoea-stage, the mother crab heads in the late afternoon for the river. At the time of twilight it enters the water, clings to a stone and beats its abdomen strongly up and down. This induces hedging of the larvae out of the egg membrane. They swim the about 100 meters of the river to its mouth and develop there in the salt water (a longer stay in fresh water is lethal for them) ([1299]). The larvae are only discharged at the time of dusk and especially at days around full- and new moon (figure 11.10). Trigger is the light-off signal. But what controls the fourteen day rhythm? To clarify it, experiments were performed.

If the animals are kept in a 14:10 hours light-dark change at 23^{0}C, the fourteen

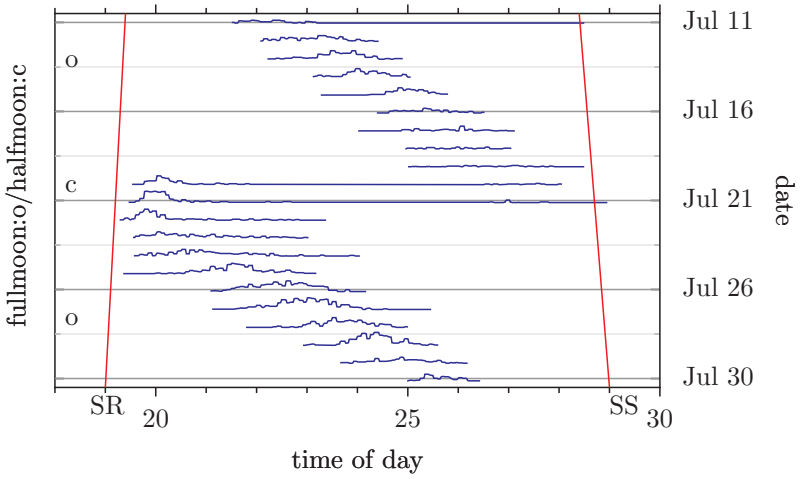

Figure 11.10: *Discharge of zoea-larvae by female terrestrial crabs of* Sesarma haematocheir. *Number of females discharging larvae: Histogram. Sun rise and sun set and moon rise and moon set are indicated together with the first and second high tide of the day. New moon, full moon, half moon. After [1298]*

day rhythm stays for about six cycles. It is thus controlled by a fourteen day clock. In a further experiment artificial moonlight was given in a 14:10 hours light-dark cycle, each night by 50 minutes later, as would occur in nature. However the artificial moon cycle was shifted against the natural one by seven days (figure 11.11, [1298]). It turned out that the artificial moon light synchronized the rhythm, under which the zoea-larvae were discharged into the water.

11.2.2 Fourteen day rhythm of *Clunio*

The small marine chironomid *Clunio marinus* is found at the European coasts of the Atlantic (a population is also found in the Baltic sea; but it has another behavior). The larvae live in algae mats in the lower most inter-tidal range. Briefly before a spring tide the mature larvae pupate. Three to five days later the males eclose at the local low tide. They fly over the area, which has fallen dry until they have found a female. They are only able to eclose from the pupal case with the help of a male (figure 11.12). They are wingless and brought by the males to favorable places, where the fertilized eggs are deposited as a jelly package on red algae.

These insects use thus a certain phase relationship between the two environmental cycles of the day (with a 24 hour rhythm) and the tides (rhythm of somewhat less than 15 days). The water level is then – because of the spring tide– especially low. Furthermore the time of low tide is chosen. This ensures that the animals eclose and mate at times when the substratum has indeed fallen dry. For the tidal events at the coasts of the Atlantic- and of the north sea see [373]. Each day the low tide and high tide occurs 50 minutes later. Every 15 days the same phase relationship and thus favorable conditions for propagation at low tide are met due to the amplitude modula-

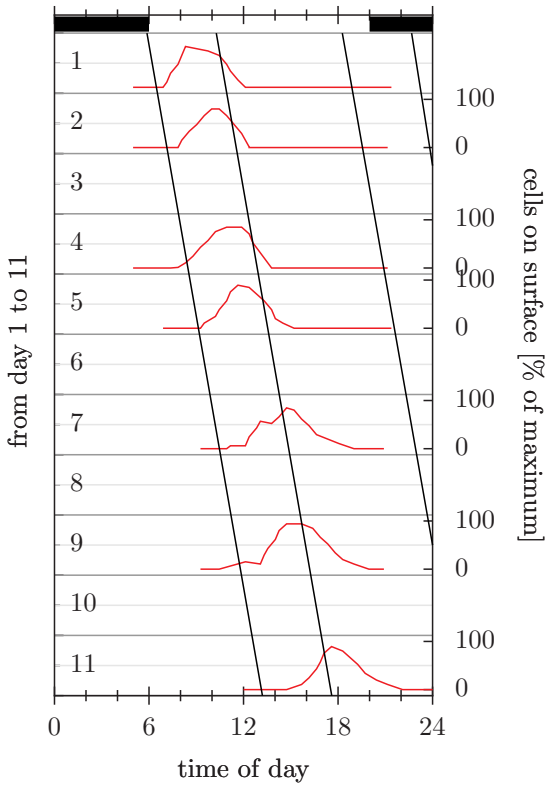

Figure 11.11: *Discharge of zoea larvae by female terrestrial crabs* Sesarma haematocheir. *The light-dark change was delayed by 6 hours as compared to the control (not shown). Whereas the control animals would discharge the larvae during the first high tide (black curve, marked high tide 1) or during the second high tide (high tide 2), they are now delayed by six hours (see red circles). Since tidal rhythms are not synchronized by the light-dark change, the shifted circadian clock must have shifted the moon clock. After [1298]*

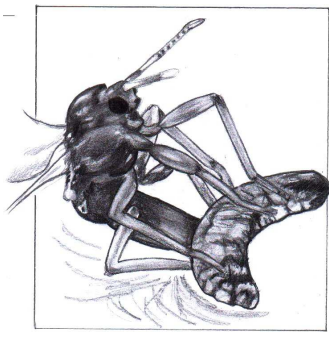

Figure 11.12: *A male of* Clunio marinus *(chironomid) copulates with a female (which are wing-less), after having cut open its pupal case with its (especially large) hypopyg of the abdomen. Drawn by Mareike Förster after [211]*

tion of the tides.

As a mechanism the animals use a circadian clock and a fourteen day clock (figure 11.13). Zeitgeber for the circadian rhythm is the light-dark change. Zeitgeber for the fourteen day rhythm is in southern populations the moon light. Northern populations however use another Zeitgeber. The summer nights in the north are too short and the moon is too low at the sky, to be able to act as a Zeitgeber. Instead water turbulence (50-200 Hz) is perceived via mechano-receptors. They have to pertain in *Clunio* for at least 6 hours, optimal for 8 hours. The end of the turbulence is most effective. The circadian system is at daytime sensitive for the change between stronger and weaker turbulence. This occurs once only every 15 days. It serves as a central nervous filter, which controls the fourteen day oscillator.

Clunio is found in several populations at the coasts. All populations eclose at the spring low tide (full moon or new moon). We are dealing with geographically isolated time-races (figure 11.14). Depend-

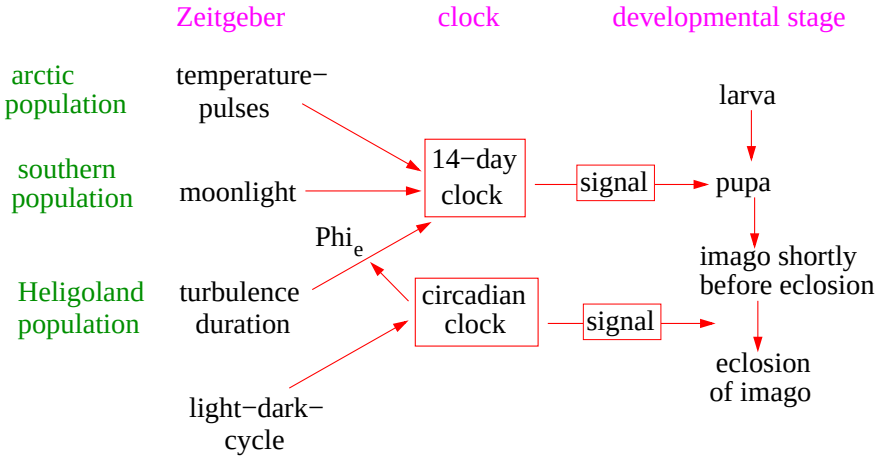

Figure 11.13: *In* Clunio marinus *pupation is controlled by signals of a fourteen day clock and eclosion of the imago out of the puparium by signals of a circadian clock. Zeitgeber of the circadian clock is the light-dark change, Zeitgeber of the fourteen day clock, depending on the population (green), the temperature change (arctic populations), the moonlight (southern populations) or the turbulence of the water (Heligoland population). Turbulence has to occur for some time (Φ_e) and to terminate at a sensitive phase of the circadian clock, in order to synchronize the fourteen day clock. After [1073] and [1077]*

ing on the location and the low tides at these particular coasts, the animals eclose at different, but specific times of the day. Crossing experiments show an intermediate heredity. It is not mono-factorial. Instead two to three genes are responsible for these time differences. Neurosecretory cells in the brain secret at certain times hormones, which determine the time of eclosion during the day.[1] The diurnal programming can change during the course of the year. In *Chironomus thumii* this is temperature dependent, in *Clunio tsushimensis* photoperiodically controlled ([1104]). There is also a flexible diurnal programming by a time of day memory ('Zeitgedächtnis´).

Physiological timing mechanisms allow also time coupling between a physiological performance and a cyclic environmental factor. It has to be reliable and the organism must possess receptors for it. The environmental conditions which are finally the time measure of this coupling and which are responsible for the selection can be quite different. For the selection conditions in *Clunio* the daylight has a Zeitgeber function (the immediately effective or ultimate factor). Selection factor for the proper diurnal phase relationship is, however the tidal cycle (the indirect or proximate factor). In Clunio-populations in the north different Zeitgeber are used: Tidal turbulence and day-night-rhythms. In populations of the south, however moon light and the day-night-rhythm are responsible. In addition non-oscillating timing systems such as turbulence longer than 6 hours are used. Arctic strains use an hourglass mechanism.

[1] Similar to the eclosion of the giant silkmoth ([1489]).

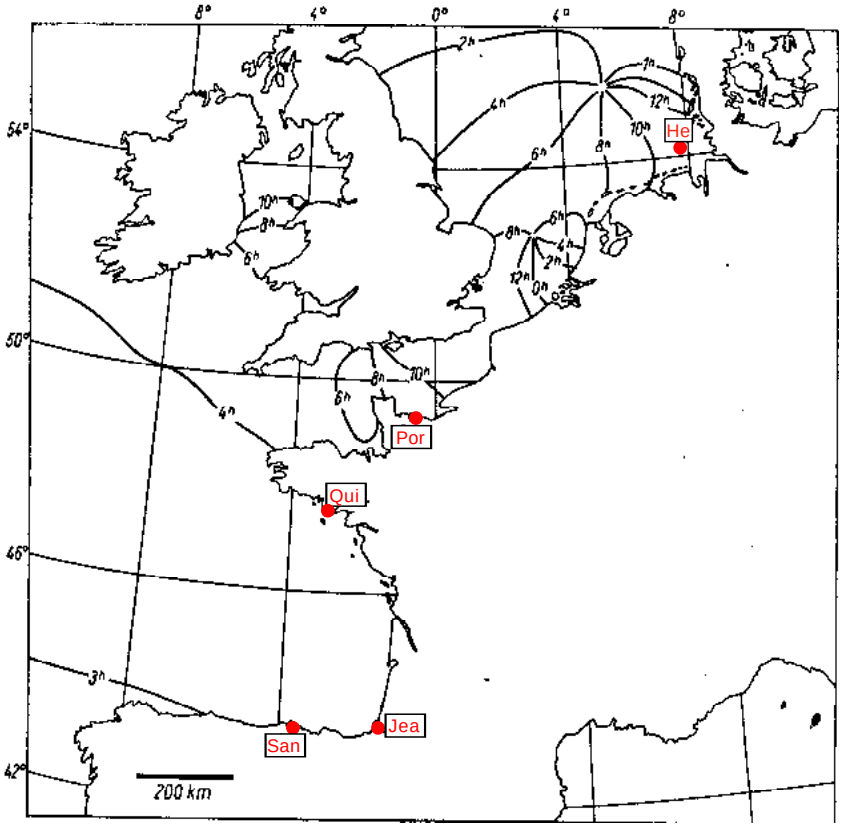

Figure 11.14: *The locations of populations of* Clunio marinus *collected from the coasts of the Atlantic and the North sea are shown (compare with figure 11.15). The collection places (German bay - Heligoland (He), Normandy - Port-en-Bessin (Por), Bretagne - Quiberon (Qui), Basque coast - St. Jean-de-luz (Jea) and coast of northern Spain - Santander (San)) are marked with red dots. The map shows furthermore the latitudes-(top) and longitudes (left) and the lines of time of high tides. These are lines of identical mean high tide differences in respect to the meridian passes of the moon in Greenwich. After [1072]*

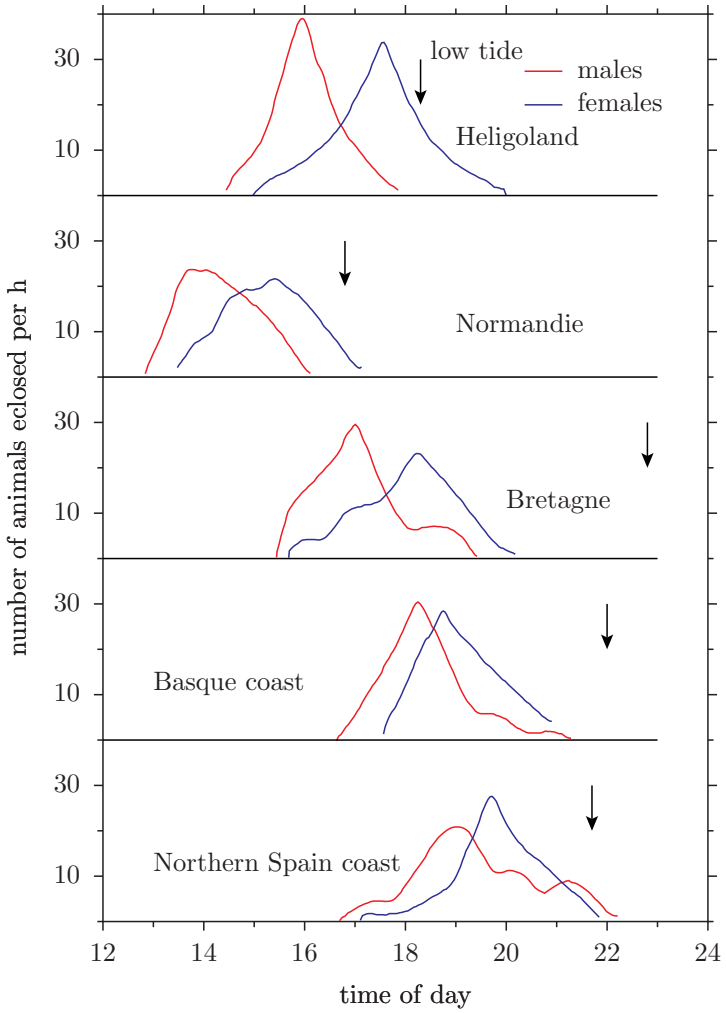

Figure 11.15: *The eclosion behavior in populations of* Clunio marinus *from the coasts of the Atlantic and the North sea are shown for males (red) and females (blue). It is in all cases a few hours before the local low tide (arrow with variation). For the collection places (German bay - Heligoland, Normandy - Port-en-Bessin, Bretagne - Quiberon, Basque coast - St. Jean-de-luz and coast of northern Spain - Santander) see map, figure 11.14. After [1072]*

They measure the temperature difference.

11.3 Monthly rhythms

In some insects monthly rhythm of activity and of eclosion of adults are observed under natural conditions. The day flies *Povilla adusta* eclose in large numbers from the lake Victoria in the days briefly before and after full moon, especially at the second day after full moon (figure 11.16). The adults live only one and a half hours. Therefore the animals have to eclose in synchrony. They do it during the short time when twilight is lengthened by the full moon. During this time the mating flight and the copulation takes place. The rhythm continues in the laboratory; it is therefore endogenous.

The pit volume of the doodle bug larvae *Myrmeleon obscurus* (Neuroptera) occurs likewise in a monthly rhythm. Around full moon the pits are large, a few days earlier small (figure 11.17). This is a true monthly rhythm, since it is observable also under continuous darkness in the laboratory. Pit and animal shown in figure 11.18.

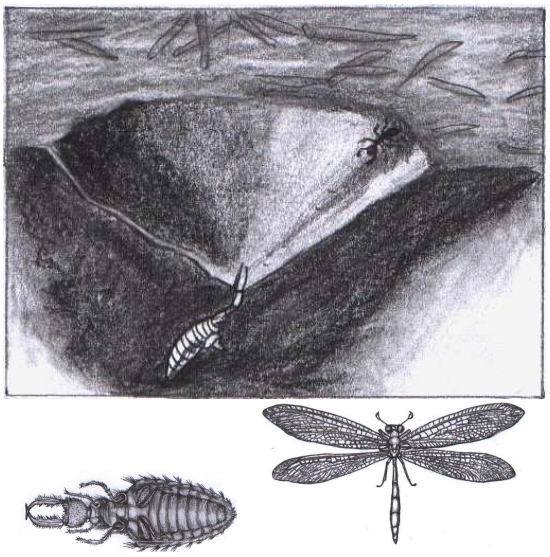

Figure 11.18: *Left: At the lowest point of the pit the larva of the doodle bug* Myrmeleon obscurus *waits for bait. Right: With its large pincers the bait is caught and digesting juice injected. Later the fluidiced interior of the bait is sucked up. Drawn by Mareike Förster after [632]*

The flight activities of bees from Morocco display a monthly rhythm during the winter and a 14-day rhythm during the summer (figure 11.19). Pollen collection of a North American solitary bee *Sphecodogastra texana* during dusk is extended into the night only if moonlight is available during this time ([754]). It is not known whether it is an endogenous lunar rhythm in this case.

Further examples are the polycheat *Typosyllis prolifera* and the Palolo worm *Eunice viridis*. *Eunice* lives in coral reefs around the Palolo-, Samoa- and Fiji-islands. The rear ends of the worm-like body containing the sexual products ('epi-

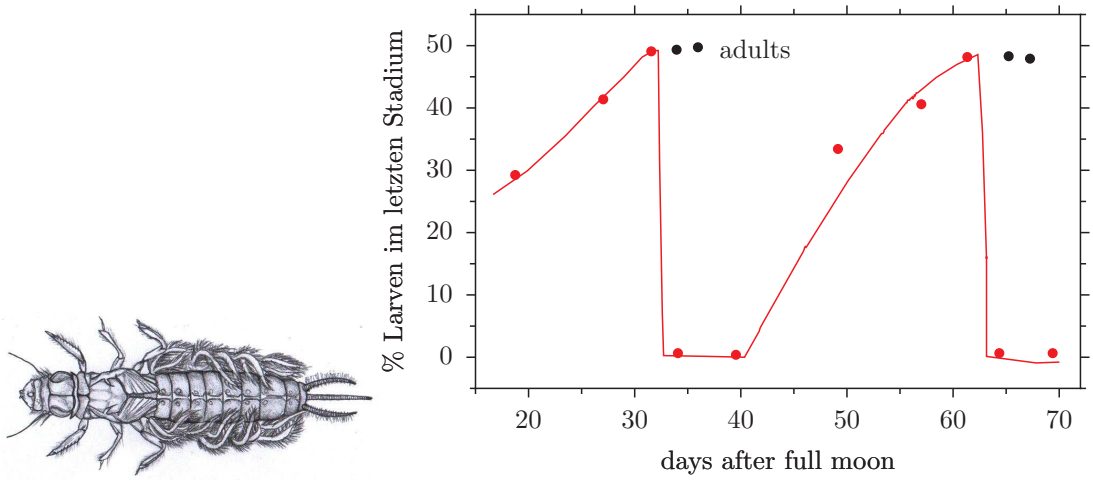

Figure 11.16: Povilla adusta *(adult: top) emerges in large numbers from the lake Victoria shortly after full moon. It is a monthly rhythm with a period length of about 30 days. After [249]*

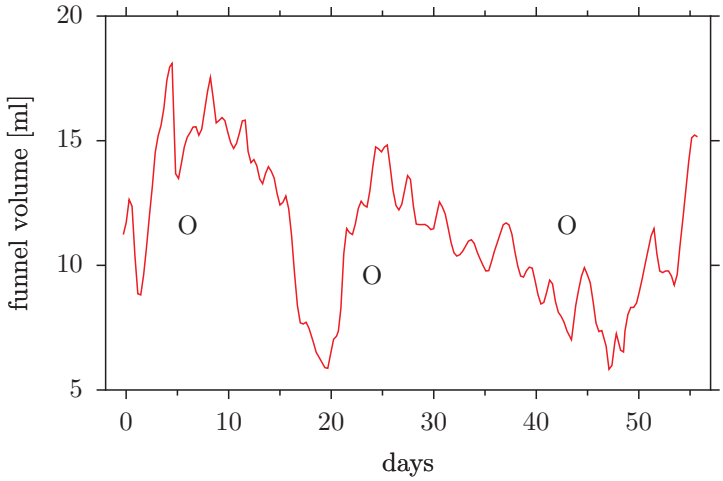

Figure 11.17: *Pit volume of the doodle bug* Myrmeleon obscurus *(Neuroptera) varies in a monthly rhythm (fuPalolo worms*
ll moon open circles). At times of full moon the pits are much larger as compared to times of new moon. Records of 24 larvae, which were kept for 55 days at 29^0C *in continuous darkness. After [1600]*

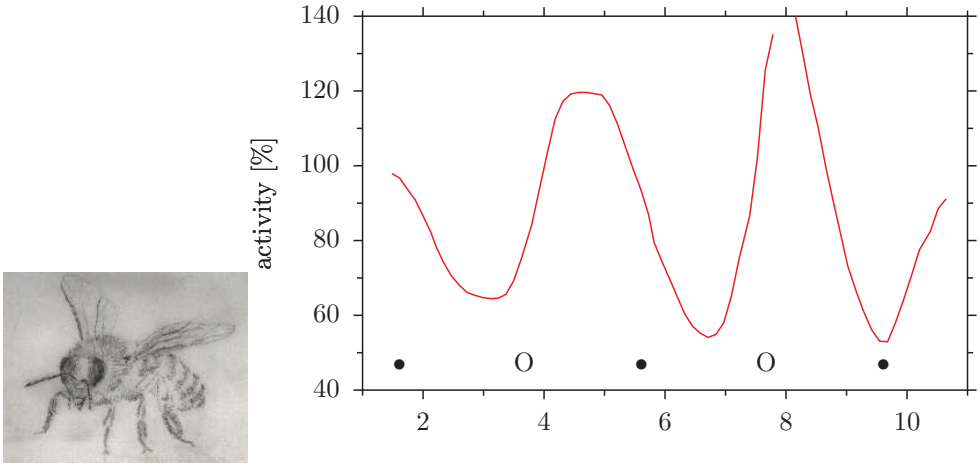

Figure 11.19: *Flight activity of bees from Marocco display a monthly rhythm during the winter and a 14-day rhythm during the summer. After [1100]*

Figure 11.20: *Palolo worms live in coral reefs around the Palolo-, Samoa- and Fiji-islands. The sexual products are discharged during the night seven days after full moon in October/November of each year and swim to the surface of the sea. The inhabitants of the islands catch them during the nights as delicious food. From [211]*

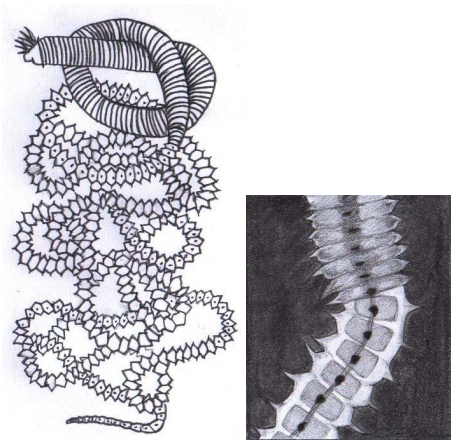

Figure 11.21: *Left: Palolo worm* Eunice viridis *(after [1129]). At the very top the 'atoke´ frontal, below the 'epitoke´ rear part of the worm-like body. Right: Close up of the transition part between frontal (top) and rear part (bottom). The sexual products (here: sperms) in the epitok are discharged and swim to the surface of the ocean. There fertilization takes place. Ventral cord runs as gray band through mid-line of body; on top of it ventral eyes as dark spots. Right figure after [585]*

tok') are discharged and reach the surface of the sea. There fertilization takes place (figures 11.20 and 11.21). This occurs during the night seven days after full moon in October /November of each year. The probability of fertilization is considerably increased in this way ([585], [211], [212]).

In the guppy the sensitivity toward colored light of yellow and violet fluctuates by about a power of one during the course of a months ([860]). The Zeitgeber is so far unknown. Air pressure differences and change in the electrical charges of the air have been excluded. Micro vibrations, fluctuations of the gravity and of the earth crust could be involved.

A book by [1131] is a good reference for tidal rhythms, rhythms with fourteen day periods and monthly rhythms. See also the books by [373] and [1145].

12 Annual rhythms

Annual rhythms help organisms, to adapt to the different conditions of the year and to prepare in time for important changes of environmental conditions during the year. In this chapter some examples for annual rhythms in organisms are presented (seed germination in plants, annual growth rhythm and emerging from cysts in algae, diapause and pupation in a beetle, body weight, gonadal weight, fur coloration and torpor in rodents, molting, migration and migratory restlessness in birds, hibernation in mammals). Afterward the properties of annual rhythms are listed. How they are synchronized by time cues and how they can be influenced experimentally, is shown in a further section. As far as known, the physiological bases of annual rhythms will be dealt with. Models of annual rhythms are given. It will be discussed, whether perhaps more than one annual clock are present in an organism. Annual rhythms are genetically fixed. This is illustrated in the example of closely related migrating birds. The adaptive significance and use of annual rhythms are treated.

The climatic conditions of the temperate and higher latitudes of the earth can fluctuate during the course of a year drastically. The temperatures during the winter are often much below the freezing point. Snow and ice prevent animals from getting food. They have to reduce metabolism or use other strategies for survival. Insects can overcome these unfavorable conditions in special resting stages. In many plants the above ground organs wilt since they can not stand frost. In all these cases it is of paramount importance that the organisms tune their development with the seasons. They must for this purpose be able to determine the time of the year in order to prepare for the coming unfavorable condition such as winter. This is often done by determining the day-length (see page 236). Day-length changes with time of the year in a regular way: Summer days have a long, winter days a short light period.

To tune the development and behavior to the seasons, an organism can also use an endogenous annual clock. In plants, for instance, an annual clock might control the germination after a time of seed dormancy. That we are indeed dealing with an annual clock, is shown by keeping the seeds under constant conditions. Even then they germinate in an annual rhythm.

In the same way as a circadian rhythm is synchronized by Zeitgeber, especially the light-dark cycle, to 24 hours, annual clocks have to be synchronized to the season. Many organisms use the length of the light period (photoperiod) or the length of the dark period for this purpose.

Annual rhythms serve as buffer systems or inert elements between environment and the physiology of an organism. They protect it against disturbances such as the weather, and smooth out short term fluctuations. Ultimate and proximate factors play a role in the control of annual rhythms. Ultimate factors act directly via favorable or unfavorable conditions such as food supply, breeding possibilities. Proximate factors on the other side

are reliable, predicting factors such as the photoperiod.

Annual rhythms in organisms are treated extensively in a book ([542]).

In addition to annual rhythms the development of plants and animals is controlled by even longer cycles, the nature of which is hardly known. There are for instance bamboo species which flower after 15 or 45 years and die afterward (as was the case in 1997/98 in bamboo of many European gardens!).

12.1 Examples for annual rhythms in plants

Annual rhythms are frequently found among plants. They can be observed in the germination of certain seeds, in the rooting of cuttings of willows, in the growth of *Lemna* and *Avena*, in change of foliage (in regions, where the rainy and the dry season change regularly, foliage renewal begins a month earlier as the rain ([182])), in the adding of new wood in stems of trees as shown by the annual rings, in the dropping of leaves in the fall, in frost hardiness, bud dormancy. Photoperiodic control of these and other events are treated in a special chapter (see page 236).

12.1.1 Seed dormancy and germination

The development of plants must be synchronized with the seasons in the temperate and higher latitudes. This holds also for dormancy and germination of seeds. The development of an angiosperm plant is shown in figure 12.1. After pollination a seed with an embryo and endosperm develops from the seed anlage. The embryo contains already all the tissue of a vegetative plant. Before developing, it enters however a resting stage. In this 'dormancy' the seeds can stand unfavorable conditions such as the low temperatures of the winter (see [1459]).

What happens during seed germination? In many plants seeds germinate if water is available allowing gas exchange. In other seeds, however, photoperiodic signals or low temperature (vernalisation) is needed for resuming development. But in some cases seed dormancy is under the control of an endogenous annual rhythm. For more information on seed germination see [591] and [819].

Endogenous annual rhythm of seed germination Seed germination can fluctuate in an annual rhythm. Bünning and coworker studied between 1940 and 1960 seeds of 335 plant species at different storage temperatures (2, 20 and 35^0C) kept in continuous darkness or continuous light. Germination was tested during the course of the year ([182]). Of these seeds 10 showed a clear annual rhythm: Maximal germination occurred for a certain species always at the same time of the year. *Hypericum, Digitalis lutea* (figure 12.2), *Potentilla molissima, Gratiola officinalis, Chrysanthemum corymbosum, Viscum album, Fragaria vesca* belong to them.

Storage temperature, water withdrawal, oxygen-, nitrogen- and CO_2 content of the air did not have any influence on the rhythm. The same was true for heat treatment. If heat treatment (110^0) was applied briefly before the germination test, however, an annual rhythm of resistance (and a high mortality rate) was observed. Under nitrogen germination was reduced, but the annual rhythm was not abolished. Under oxygen the germination was increased and again an annual rhythm found.

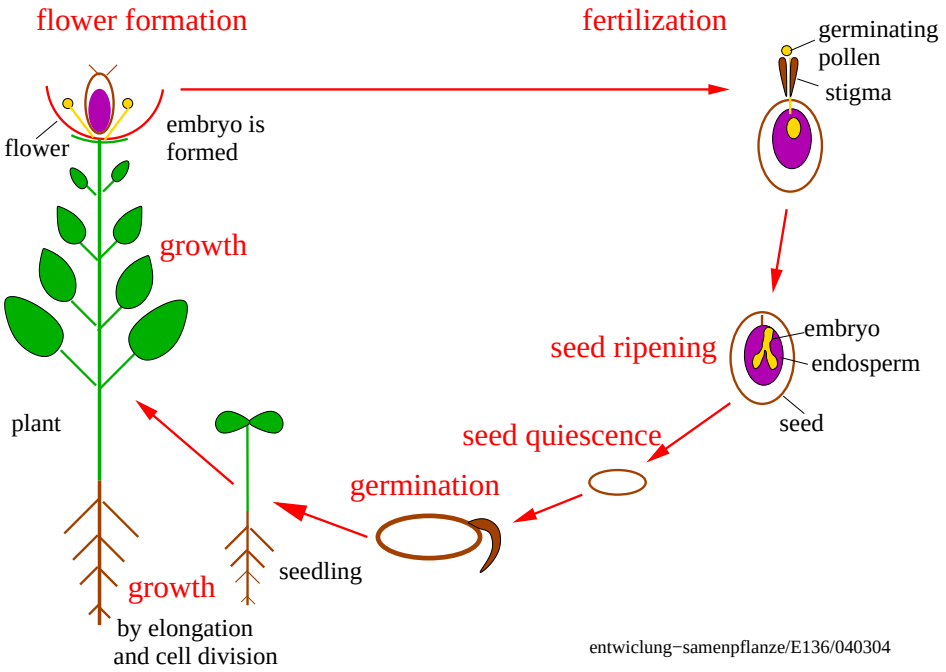

Figure 12.1: *Life cycle of an angiosperm plant: Embryo formation, ripening, dormancy, germination of the seed, development of the seedling, development of the plant, flower formation and fertilization*

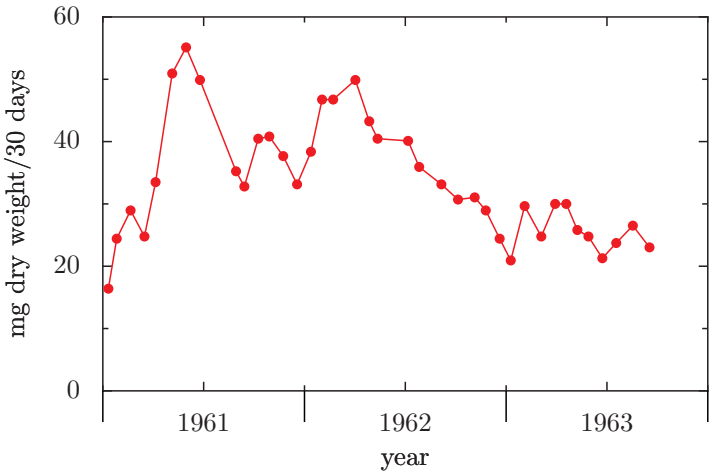

Figure 12.3: *Annual rhythm of substance production rate in* Lemna. *After [133]*

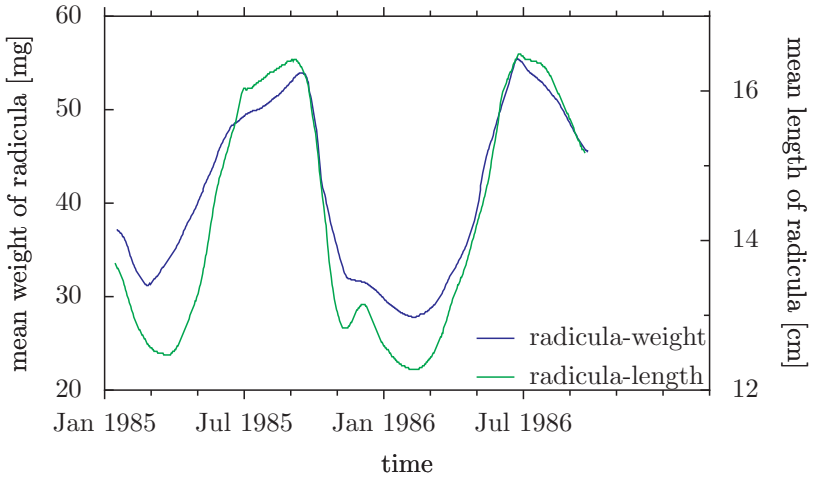

Figure 12.4: *Annual rhythm of radicle length (blue) and radicle weight (green) in bean seedlings. After [1405]*

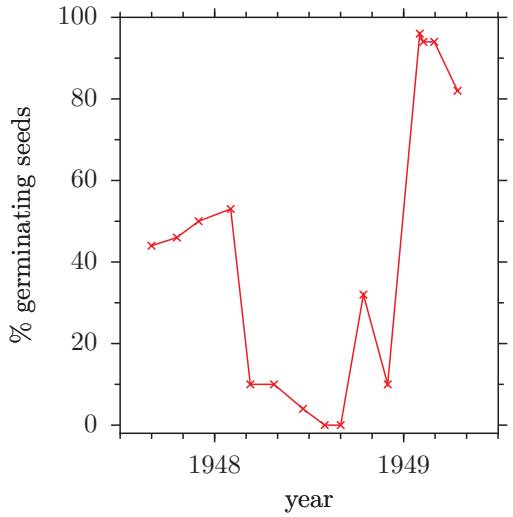

Figure 12.2: *Annual rhythm of seed germination of* Digitalis lutea. *Seeds were stored at* 3^0C *and around every 45 days placed on wet filter paper at* 23^0C *in continuous darkness. The percentage of germinated seeds is plotted against the time of the year. After [181], figure 6*

In dry seeds an annual rhythm of respiration was found. It runs parallel to an annual rhythm of water permeability and swelling. The physiological cause of this annual rhythm is unknown (figure 12.3). Spruyt and coworker studied the water uptake and enzyme activity of bean seeds (figure 12.5, [1405], [1406]). The seeds were imbibed for 4 hours in water and afterward the weight determined. Germination correlated with water uptake. Water uptake as well as root length and weight fluctuated in an annual rhythm with a period length of 10 to 11 months (figure 12.4).

During dormancy the elongation of the embryo is inhibited ([862]).

In *Fragaria* the mother plant influences the germination of the seed: Seed, which ripened at different times of the year, was harvested and the germination rate determined in the following months. It reached, independently of the time of harvesting, in all samples a maximum in October (figure 12.6). How the annual rhythm of seed germination is synchronized, is unknown.

The role of photoperiodic reactions in

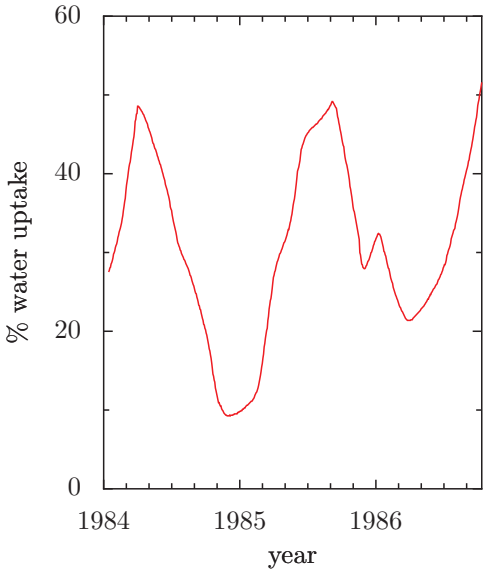

Figure 12.5: *Annual fluctuations of water uptake (in percent of dry weight) of dry bean seeds (*Phaseolus vulgaris*) during four hours at* 25^0C *in darkness, measured from June 1984 to July 1986. After [1405]*

seed dormancy and seed germination will be treated later (see section 13.2.4).

12.2 Annual rhythms in algae

Annual rhythms have been described in a number of algae. Two examples are given, the annual growth rhythm of the phylloids of *Laminaria* and the emergence of the diatom *Alexandrium tamarense* from cysts in the spring.

12.2.1 Annual rhythm of the phylloid growth in *Laminaria*

Laminariales are large brown algae (*Phaeophyceae*) of oceans. Their sporophytes consist of a rhizoid, a cauloid and a phylloid. The leaf-like phylloids grow mainly during the winter by using the reserves of the

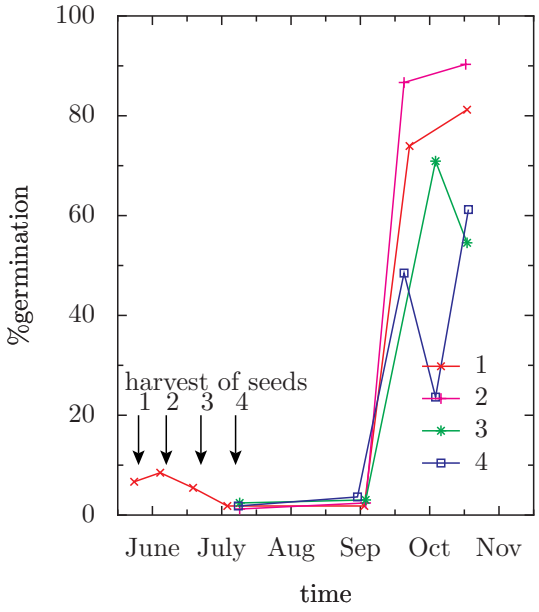

Figure 12.6: *Readiness of seeds of* Fragaria vesca *to germinate. The seeds ripened at different times of the year. In spite of it, independent of the harvest time (arrows with different colors) readiness to germinate was in all samples (different curves) highest during October ([181])*

old phylloid ([932]). It was found that the phylloids of *Laminaria hyperborea*, *digitata* and *Pterygophora californica* grow in an annual rhythm (figure 12.7 and [1327], [935]). This rhythm was found also in cultures, which were kept in containers under constant day-length and temperature. The nutrient supply was uniform. Under these conditions the period length of the annual rhythm was 33 to 40 weeks in *Laminaria hyperborea* (12 hours light per day) and 27 to 34 weeks in *L. digitata* (8 hours light per day), that is, shorter as a year. The annual rhythm is only found under long day (critical day-length 8-9 hours). Under short day no annual rhythm is found, but with a light pulse during the middle of the dark period the annual rhythm is again obtained[1] (it is known that short day with a light break during the night acts like a long day in photoperiodic reactions).

The phylloid growth can be synchronized by modulating the day-lengths in a sinusoidal or rectangular way in a cycle of 12 months, but also with 6- and even 3-month cycles (figure 12.8 and [934]). The annual rhythm has thus a large range of entrainment, as is known also from other objects with annual rhythms. Long day with light periods exceeding 8 hours synchronize the circannual rhythm to the natural year. In *Agarum cribrosum*, *Pleurophycus gardneri*, *Laminaria saccharina*, *L. bongardiana* and *Desmarestia aculeata* the growth can also be synchronized by artificial day-lengths. But in these algae a circannual growth rhythm has not yet been demonstrated.

[1]The period length is, however, only 20 to 26 weeks.

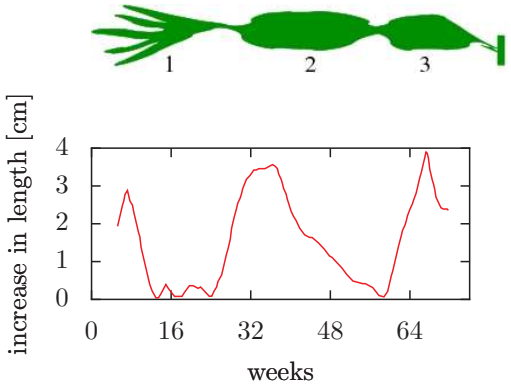

Figure 12.7: Pterygophora californica *sporophyt (top) with rhizoid (right), cauloid (stalk-like) and phylloid (left). Phylloids grow mainly during the winter and form at the base a new 'leaf' using storage products of the old one. The annual rhythm of this growth is shown in the lower part. 1 (top) is the oldest (first growth incidence in the curve), 3 the youngest (third growth incidence in the curve). After [934]*

12.2.2 A marine algae with an internal calendar

The diatom *Alexandrium tamarense* possess an annual rhythm. A related species was already mentioned while presenting its circadian control of bioluminescence (page 97). In *Alexandrium tamarense* the algae emerge in the spring from cysts. This event was studied in detail ([16]):

The species is a danger in coastal waters of the oceans: The algae might bloom. A toxic substance is produced which poisons fish. Fishing has to be stopped, because humans are also poisoned in eating these fishes. In the golf of Main at the eastern coast of the United States such episodes are April to November. During this time the cells are vegetative and move with two flagella. During the winter they sink to the bottom of the sea, cast the shells and flag-

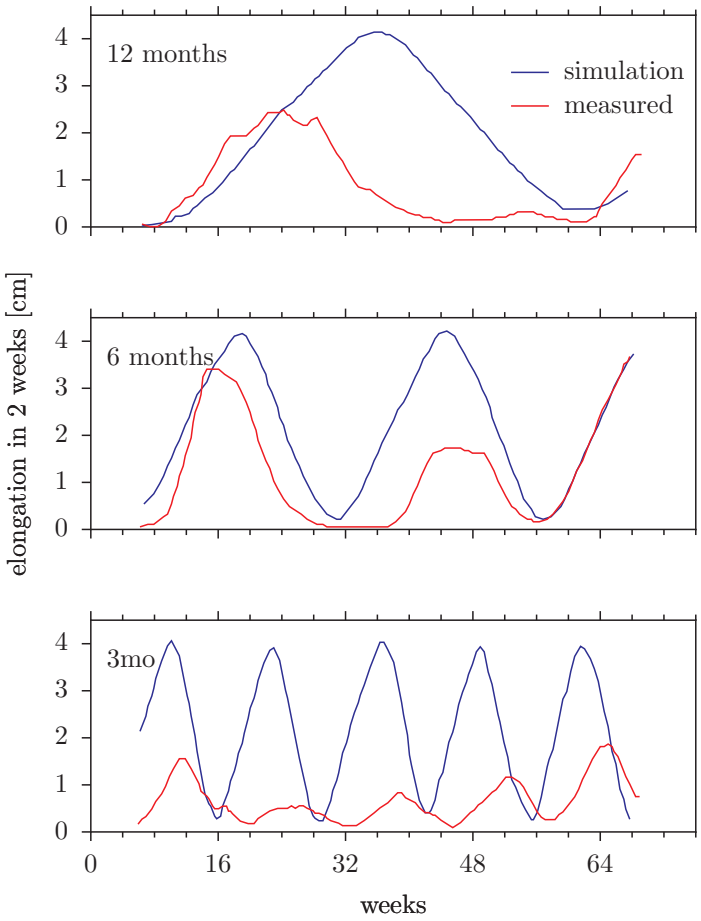

Figure 12.8: *Synchronization of the annual rhythm of growth rate of the terminal phylloids of* Pterygophora californica *by modulating the day-lengths in a sinusoidal way (12, 6, 3 months-cycles). The annual change of day-length at* 54^0 *N latitude was simulated. Temperature* 5^0*C. After [934]*

ella and form cysts, in which they overwinter for 2 to to 6 months (dormancy). In the spring the algae eclose from the cysts, form a new shell and flagella and arrive at surface waters of the sea.

Eclosion from the cysts is controlled by an annual rhythm: If samples of algae are taken from the sediments of the bottom of the sea at different times of the year and transferred in a 14:10 hours light-dark cycle at 15^0C in the laboratory, algae eclose in an annual rhythm as shown in figure 12.9. This rhythm is also found, if a larger sample, collected in August, is kept in a refrigerator at 2^0C and samples of it taken from the refrigerator at different times of the year into 15^0C. The results are shown in figure 12.9. Since the oscillation continuous for two years, we are dealing with a true annual rhythm. Algae populations in flatter coastal waters do not show this annual rhythm. They might obtain seasonal time cues and do not need to rely on an endogenous rhythm.

Annual rhythms are also known from other dinoflagellates and they are of ecological significance ([263]).

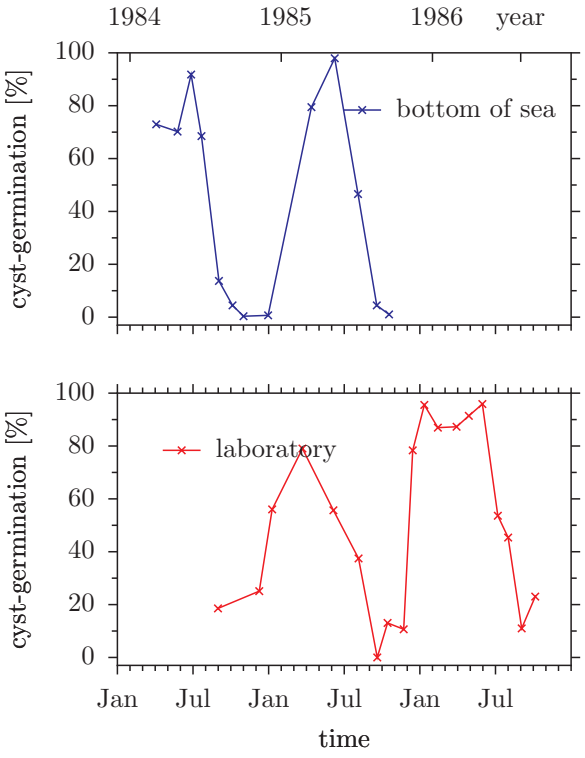

Figure 12.9: Alexandrium tamarensis *from the Golf of Main eclose in an annual rhythm from the cysts. Top: Percentage of germination of cysts in samples taken from the bottom of the sea. Bottom: A larger sample from the bottom of the sea was kept at 4^0C in a chilling room. Samples of it were taken and transferred into 18^0C at different times during the course of two years. The percentage of germinating cysts is plotted as a function of the time of the year. After [16]*

12.3 Further examples for annual rhythms in plants

Further annual rhythms were observed in plants (in some of them the endogenous character has not been tested yet).

- Nitrate reduction in *Ankistrodesmus braunii* in continuous light at 23^0C ([759]),
- Development in mosses ([696]),
- Growth, dry weight, protein content, and carbohydrate content in *Lemna minor* ([133]),

- Perennial organs in water plants ([619]).
- *Salix*-cuttings root differently well during the course of a year, if treated with IAA (0.01 to 0.001%) (figure 12.10). In *Populus* two peaks per year are observed ([535]).
- Germination and leaf movement of *Oxalis regnellii* and *Oxalis acetosella* (both shadow plants) fluctuates in an annual rhythm ([1046]). Period length amounts to 13 months ([837]).
- Elongation of primary leaves of seedlings in *Avena sativa* fluctuates in an annual rhythm ([904]).
- The shoot- and root system of *Symphytum officinale* develops during the year differently. Carbohydrate content and amount of fructosanes fluctuates in an annual rhythm ([1407]).
- The production capacity of grass fluctuates annually ([1201]).
- An annual rhythm was found in wheat for the time until flowering ([701]). In all spring varieties early germinating plants did flower earlier as late germinating plants. This was independent of the year of harvest and the age of the seed, the localization, the nitrogen supply. It is likely that this flowering of spring wheat is controlled by an endogenous annual rhythm. It should be taken into account if experiments are made at different times of the year.
- Annual rhythm of bud dormancy ([662], [266]).
- Stomata movement of beans fluctuates in an annual rhythm in beans.

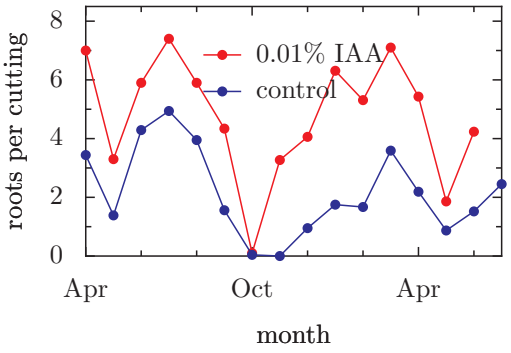

Figure 12.10: Salix-*cuttings root more or less well, depending on the time of the year. They were all treated with IAA (0.01 to 0.001%). After [535]*

In these experiments the air was filtered, to avoid chemical signals which might undergo an annual change ([1361], figure 12.11).

12.4 Annual rhythms in animals

In the preceding section some annual rhythms in algae and plants were described. Annual rhythms are, however, much better known in animals. They are especially well studied in birds and mammals ([30], [542]). Reproductive activity, body weight, fur- or feather change, bird migration are examples. Hibernation as a survival strategy of some mammals is also controlled by an annual rhythm (see section 12.4.4).

The changes during the seasons are much more drastic as those during a day. This is the reason why physiological conditions and behavior of animals are much more fundamentally changed by annual rhythms as they are by daily rhythms.

In the next sections an example for annual rhythms in invertebrates, namely the

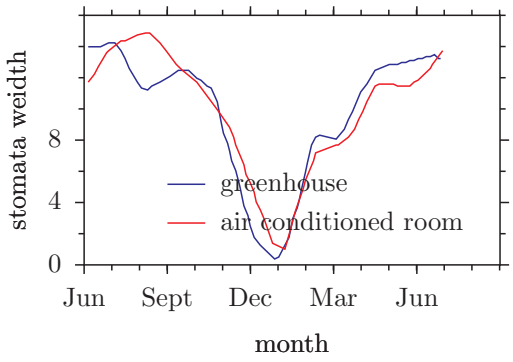

Figure 12.11: *Annual fluctuations of stomata width of bean plants kept in a greenhouse (stippled curve) and in an air conditioned room (solid curve). In the greenhouse air pressure, light intensity, temperature and relative humidity change during the course of a day and the year. In the air conditioned chamber temperature, light intensity and photoperiod were kept constant (16:8 hours light-dark-change). The air was purified using carbon filter. The CO_2-concentration was less than 1ppm. After [1361]*

carpet beetle *Anthrenus verbasci* and three examples of annual rhythms in vertebrates are presented:

1. The annual rhythms of the Dsungarian hamster (reproduction, body weight, gonadal weight, fur coloration).

2. The annual changes in birds (migration, body weight, activity, molt and reproduction, see 12.4.3)

3. Circannual control of hibernation in mammals.

12.4.1 Annual rhythms in invertebrates

Many properties, behaviors, physiological events and developmental steps of insects fluctuate during the course of a year. Whether we are dealing with a circannual rhythm can, however, be decided only if experiments are made under constant conditions.

In insects only a few cases of a true annual rhythm have been observed if these criteria are used. One of it is the carpet beetle *Anthrenus verbasci* (*Dermestidae*). This beetle destroys often valuable zoological collections. It is a long living insect, which survives the first and second winter as a larva in a diapause stage (for diapause see 13.3). Afterward the larvae pupate. In the following spring the adults eclose. In a population which was kept in continuous darkness, [107] observed an annual rhythm of diapause and pupation (figure 12.12). At temperatures above 20^0C all animals of a population are already eclosed in one year. The period length of the annual rhythm can therefore not be determined. At 17.5^0C and 20^0C eclosion is spread over two years. The period length lies between

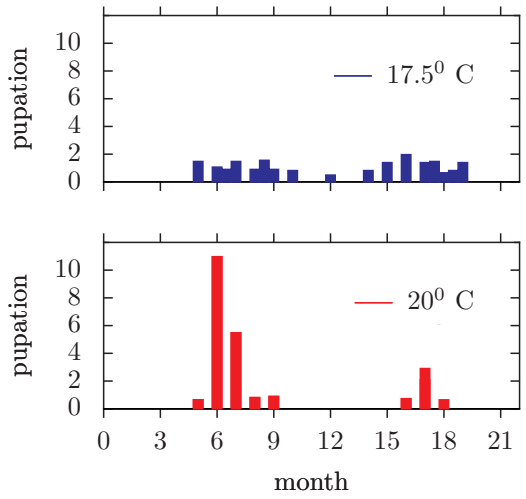

Figure 12.12: *Annual rhythm of diapause and pupation of* Anthrenus verbasci *(Dermestidae), a pest of zoological collections. In continuous darkness, constant temperature of* 20^0 *and* 17.5^0C *and constant humidity of the air some larvae pupate during the first, others during the second 'gate'. The distances of the occurrences of these pupations are 10 to 11 months. Thus we are dealing with an endogenous annual rhythm. After [107]*

10 and 11 months. The annual rhythm allows eclosion only at certain times of the year. Animals, which were not far enough developed, have to wait until the eclosion gate of the next year is open.

Recently the annual rhythm of the carpet beetle has been re-investigated and extended using a Japanese population ([1093]). The temperature compensation was re-confirmed and transfer from long day conditions to short day conditions shown to be the Zeitgeber for the circannual rhythm.

The slug *Limax flavus* was kept under constant temperature (20^0 respectively 10^0 C) and humidity ($40-60\%$). It lays eggs in a circannual rhythm, as shown in figure

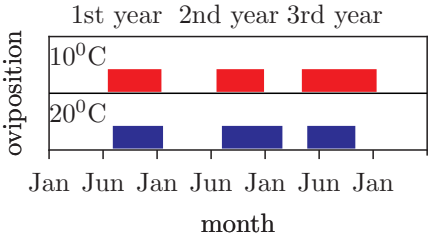

Figure 12.13: Limax flavus *kept under constant temperature of* 20^0 *and* 10^0C *and a relative humidity of* $40-60\%$ *show a circannual rhythm in egg deposition. After [1358]*

12.13.

12.4.2 Annual rhythm in the Dsungarian hamster

Annual rhythms are known from several mammals, such as the golden mantled ground squirrel *Spermophilus lateralis* ([1147]), the squirrel *Tamiasciurus hudsonicus* ([73]), bats ([282]), sheep (annual rhythm of wool growth ([1218]), rectal temperature of Corriedale-sheep under tropical conditions ([291]), rhesus monkeys and more (see table 2.1 in [542]).

In the Dsungarian hamster (*Phodopus sungorus*) the seasonal changes of the environmental conditions are especially extreme. The air temperatures might rise in its natural habitat in the summer to 45^0C and drop during the winter to -64^0C. It is therefore to be expected, that the animals adapt to these changes in respect to their physiology, morphology and behavior. The body weight, gonadal weight and fur coloration (figure 12.14) fluctuate indeed in an annual rhythm (figure 12.15 and [645]). It restricts reproduction of these animals to a certain season, which increases the chances of the offspring to survive.

Likewise reproduction of the Syrian

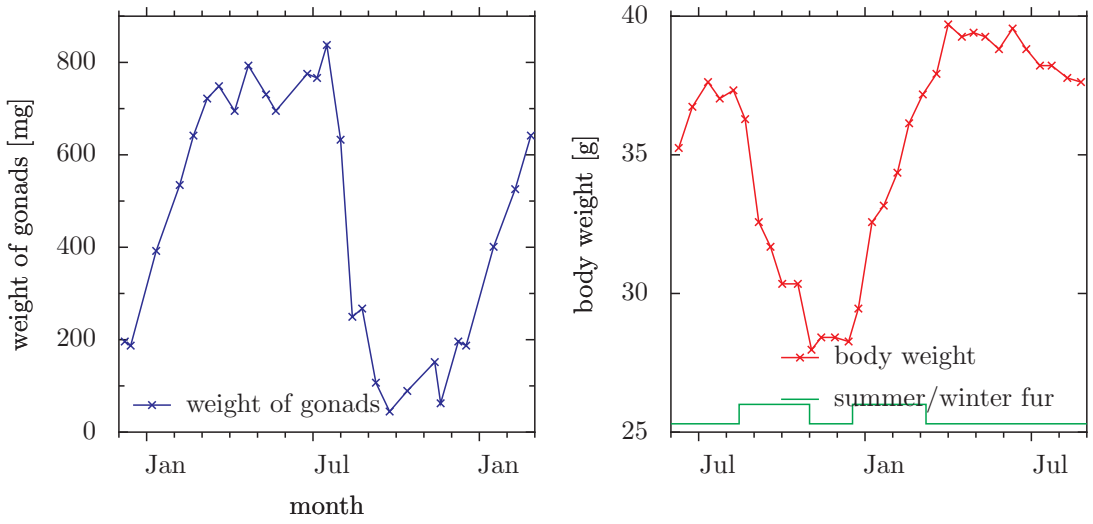

Figure 12.15: *Annual rhythms in the Djungarian hamster* Phodopus sungorus. *In the late summer and fall the body weight decreases (top, red) and the gonads regress (bottom). Regression is terminated after the animals have been for some time under short day conditions: The gonads develop again, body weight increases* (recrudescence). *After [646]*

Figure 12.14: *Dsungarian hamster* (Phodopus sungorus) *in summer- (left) and winter fur (right). After [428]*

hamster ([513]) and the European hamster ([966]) is not only photoperiodically controlled, but underlies a circannual rhythm. In the Dsungarian hamster the annual component is, however much more expressed. Photoperiod is perceived by the mother and signals passed to the fetus ([1228]). They determine the reproductive pattern of the offsprings after they have grown up.

Photoperiodic control and annual rhythms serve to adjust the reproductive and non-reproductive periods to the season with the most favorable conditions. An endogenous annual rhythm by itself would not be sufficient, to restrict the necessary physiological events precisely enough to a certain narrow time span. An additional photoperiodic control synchronizes the endogenous annual rhythm with the annual rhythm of the environment. In this way it is attained in the Dsungarian hamster that all males develop sperms at the same time and that shortly afterward all females have their estrus. This ensures reproduction of the animals reliably.

12.4.3 Annual rhythms in birds

Annual rhythms have been studied quite intensively in birds. Mainly events connected with migration to the winter quarters and the summer quarters and with reproductive behavior such as change of

the feathers (molt), body weight and food preference, gonadal growth and migratory behavior are under control of this clock.

Yearly about 600 million birds migrate to their breeding or winter quarters ([153], [99]). Migration covers each year many months, whereas breeding can be quite short (one month). Therefore a precise time schedule is needed. This time schedule exists in form of an endogenous timing program. It is genetically fixed. Short distance migrants can be more flexible. Therefore their departure time and arrival time vary more strongly. They migrate nine months and breed just one month.

How precise these annual rhythms function is shown by so called 'calendar birds', which arrive on certain days of the year in their summer quarters in higher latitudes. The sand piper for instance appears in Helsinki (Finland) between the first and eights of May (4.5 ± 2.06 d), the Northern cliff swallow *Pterochelidon albifrons albifrons* arrives in San Juan Capistrano, California, around the 19th of March (figure 12.16).

In tropical forms and trans-equatorial migrants annual rhythms are useful, because the differences in day-length are too small in the areas close to the equator. Photoperiodic signals are not useful anymore to trigger migration time.

The synchronization with the environment uses a flexible system consisting of an internal annual clock and time cues of the environment such as photoperiodical signals, but additionally also fine regulators. Together they provide high flexibility and adaptability on the one hand, and high reliability on the other hand.

Bird migration, migratory restlessness, molting

Many animals have a drive to migrate for instance in searching for food. They come during the winter from the mountains down into the valleys or during the hot season from the Savannah to wetter areas. In birds these movements are much more pronounced. Often migration begins already while plenty of food is still available (golden oriole, swifts). Before migrating they become restless ('migratory restlessness'). Long distance migrants cover huge distances between the winter quarters and summer quarters. The arctic tern for instance migrates twice per year a distance of 10 000 km. The swallow migrates in the fall to southern Africa and back in the spring. Even small birds such as the rubinthroated hummingbird take long routes. This bird crosses the golf of Mexico. It weights normally 2g only. Before migration it adds another 2g of weight. During migration many animals use the sun and/or stars (see chapter 10).

First indications of an annual control of bird migrations were obtained from observations of the willow warbler (*Phylloscopus trochilus*, [540]). They stay for a long time at equatorial regions. In March they start to migrate to higher latitudes, during the fall (late July, August) they migrate to its equatorial winter quarters. Like many other small migrating birds it migrates during the night, although it is normally a day-active bird. If kept in cages, it develops migratory restlessness around this time (figure 12.17).

Before migration begins a number of preparations are made: The feathers are changed (molting). Fat is deposited. This increases the body weight considerably. It was first assumed that these events are

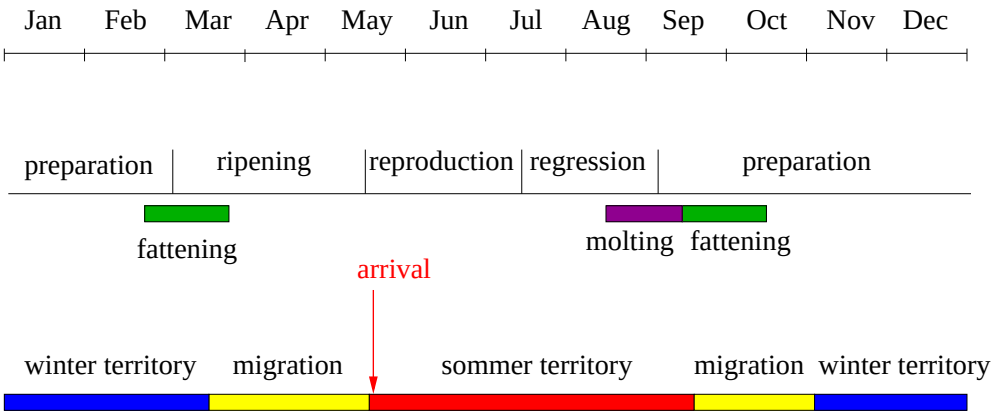

Figure 12.16: *Typical annual breeding and migratory cycle of birds of temperate zones. Upper row shows events in the bird, lower row the time spent in the summer respectively winter territories (x-axis month). In calendar birds the arrival time in the summer territory is confined to a few specific days of a certain month. After [71]*

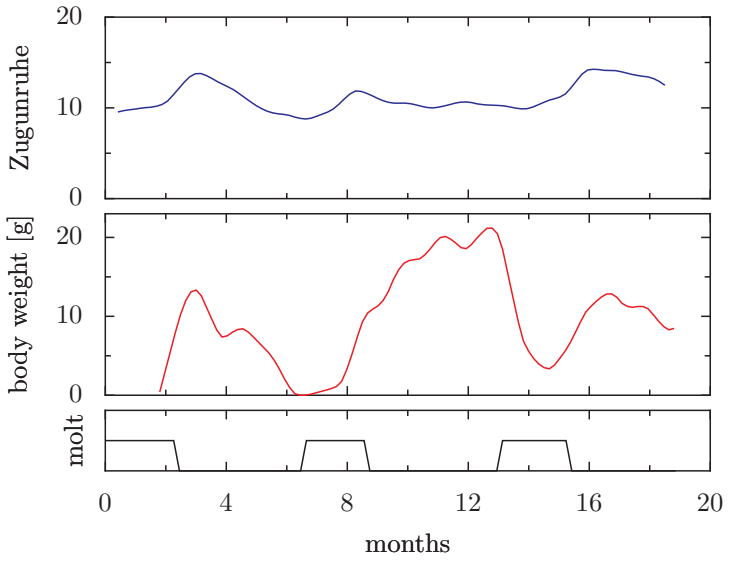

Figure 12.18: *Circannual rhythm of nocturnal activity (curve) and molt (bar) of a willow warbler (*Phylloscopus trochilus*), which was kept for 28 months under constant temperature and 12:12 light-dark-cycles. The number of nocturnal ten minute-intervals with activity is plotted as a function of the time of the year (months). After [539]*

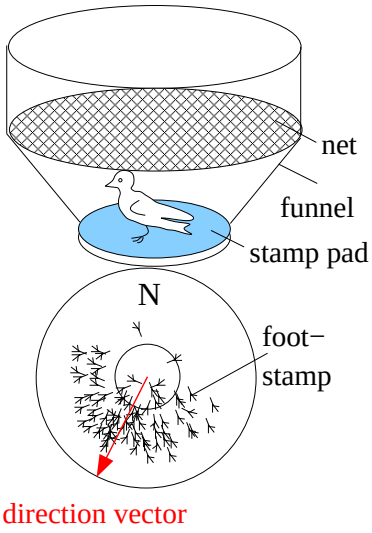

Figure 12.17: *Top: An Emlen-cage is used to record migratory restlessness. An ink-pad in the center colorates the feet of the bird. During migratory restlessness the paper covering the funnel is blackened in the preferred direction of attempted take offs of the bird. Net and blind covers the funnel. Bottom: Circular histogram showing amount and direction of jumps. After [371]*

induced by the day-length. The differences in day-length are, however, in the vicinity of the equator quite small. Therefore it is not too astonishing, that these changes in the physiology and in behavior occur also, if the animals were kept for longer time periods in the laboratory under the same day-lengths (12:12h light-dark-change). After 28 months of recording a curve was obtained for nocturnal activity as shown in figure 12.18. A circannual rhythm of 10 months was found ([539]). This shows that even without external time cues an endogenous annual program proceeds, which controls the preparation for migration and the migration itself. Since the 'free run period' deviates clearly from the length of a year (10 instead of 12 months), we are dealing with an endogenous rhythm.

Similar studies were done with warbler. Figure 12.19 shows the changes of gonadal size in the garden warbler (*Sylvia borin*) during 33 months at a constant temperature of 20^0C and under continuous light-dark-changes of 12:12 hours compared with natural day-night conditions ([100]). Black caps (*Sylvia atricapilla*) were kept for more than 8 years in a light-dark-change of 10:14 hours ([98]). They too exhibited an endogenous annual rhythm of molt. The period length was 10 months. Thus nine endogenous years occurred during the 8 years of observation.

Circannual rhythms of gonad development were studied in detail in starlings (*Sturnus vulgaris*) ([541]). Figure 12.20 shows the results on experiments in which animals were kept for 43 months either in a 12:12 hour light-dark-change (upper part of the figure) or in a 11:11 hour light-dark-change (lower part of the figure). In both cases the size of the gonads and the times of molting were under circannual control.

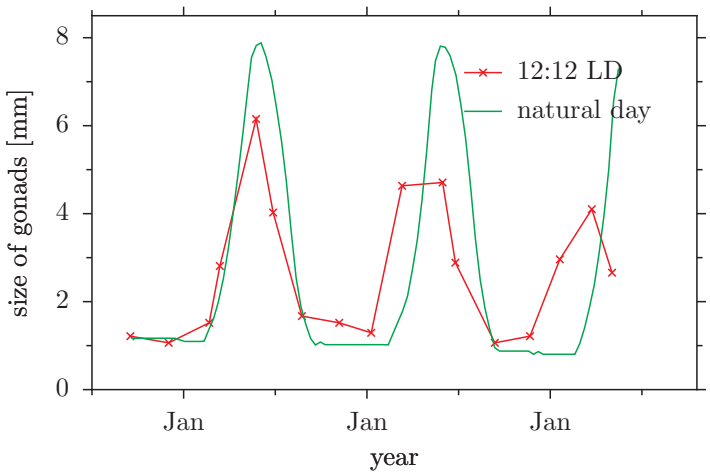

Figure 12.19: *Annual rhythm of gonadal length of a garden warbler (*Sylvia borin*) during the course of 32 months at a constant temperature of* 20^0*C. One group (green curve) was kept under natural day conditions, a second group (red curve) under a 12:12 hour light-dark-cycle. January of each year marked. After [100]*

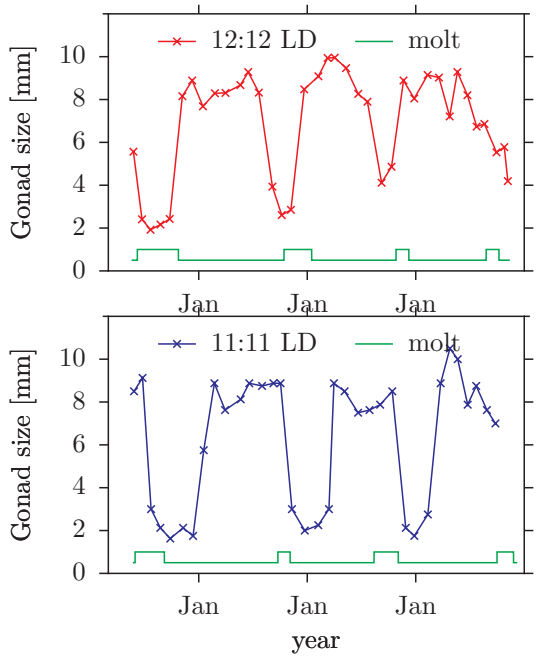

Figure 12.20: *Circannual rhythm of gonad size (curve) and of molt (bar) in the starling (*Sturnus vulgaris*). The animal was kept for 43 months individually either in a 12:12 hour light-dark-cycle (upper curve, red) or in a 11:11 hour light-dark-cycle (lower curve, black). After [541]*

The different day-lengths (24 hour respectively 22 hour days) did not matter.

In the starling it was also shown, that the conditions under which an endogenous annual rhythm occurs in the laboratory are limited: Neither in a 11:13- nor in a 13:11 hour light-dark-cycle a circannual rhythm is exhibited, but it is found under a 12:12 light-dark-cycle (figure 12.21) .

If the animals are transferred from a 11:13 hour light-dark-cycle into a 12:12 hour light-dark-cycle or from a 13:11 hour light-dark-cycle into a 12:12 hour light-dark-cycle, gonadal development and molting start anew with a fixed interval to the onset of the 12:12 hour light-dark-cycle. This shows that the circannual clock was stopped and not just uncoupled from its hand or masked by an other effect which superimposed the annual rhythm. Cause of the arrest of the annual rhythm under 11:13 hour light-dark-cycles is the short day: Only long days are able to release the refractory state which was induced by the short days. Cause of the arrest of the annual rhythm under 13:11 hour light-dark-cycles is the long day: The animals can not enter the refractory state, because this is only possible under short day conditions. In a 12:12 hour light-dark-cycle, however, both events can occur: The refractory stage can be induced *and* terminated.

12.4.4 Examples for hibernation

Animals in the temperate and higher latitudes of the earth are exposed to low temperatures and food shortage during the winter. They need therefore special strategies to survive. Hibernation is such a strategy of certain mammals. During hibernation a considerable amount of energy is saved by these animals. In fact, this energy saving might have been an ancestral capacity and used also in mild climates with enough food available. In more severe climates and with food shortage mammals and marsupials specialized later in evolution to survive harsh conditions. Hibernation in mild climates corresponds to thermoregulation in reptiles and is found in some birds (mouse-birds, *Coliiformes*, [979]), marsupials (*Echidna*, [529] and [1085]) and eutherian species ([925]).

A recent report on an international symposium ([595]) and a review article ([820]) are recommended for details. The term 'torpor´ is used to describe different occurrence of hypo-metabolism, hypothermia, and behavioral arrest. Hibernation and aestivation are used to describe seasonal aspects, whereas daily torpor (see page 287) is of a circadian nature (see figure 12.25).

Among hibernating mammals are *Chiroptera* (bats), *Rodentia* (hamster, marmots, door-mouse and other *Myoxidae*) and *insectivors* (for instance the hedgehog). Using a few examples, hibernation is illustrated in the following. A number of questions are pertinent such as: How is hibernation induced, maintained and terminated? What are the characteristics of hibernation, which internal and external factors are involved, what is the physiological basis of it? Which control centers are involved? Which are the theories and models concerning hibernation?

The golden mantled ground squirrel *Spermophilus lateralis* (=*Citellus lateralis*?) lives in the western parts of north America from British Columbia to California in a heights of 1500-3600m. The animals are under natural conditions obligatory hibernators (figure 12.22). They stay under this condition for many months in their burrows in continuous darkness and more or

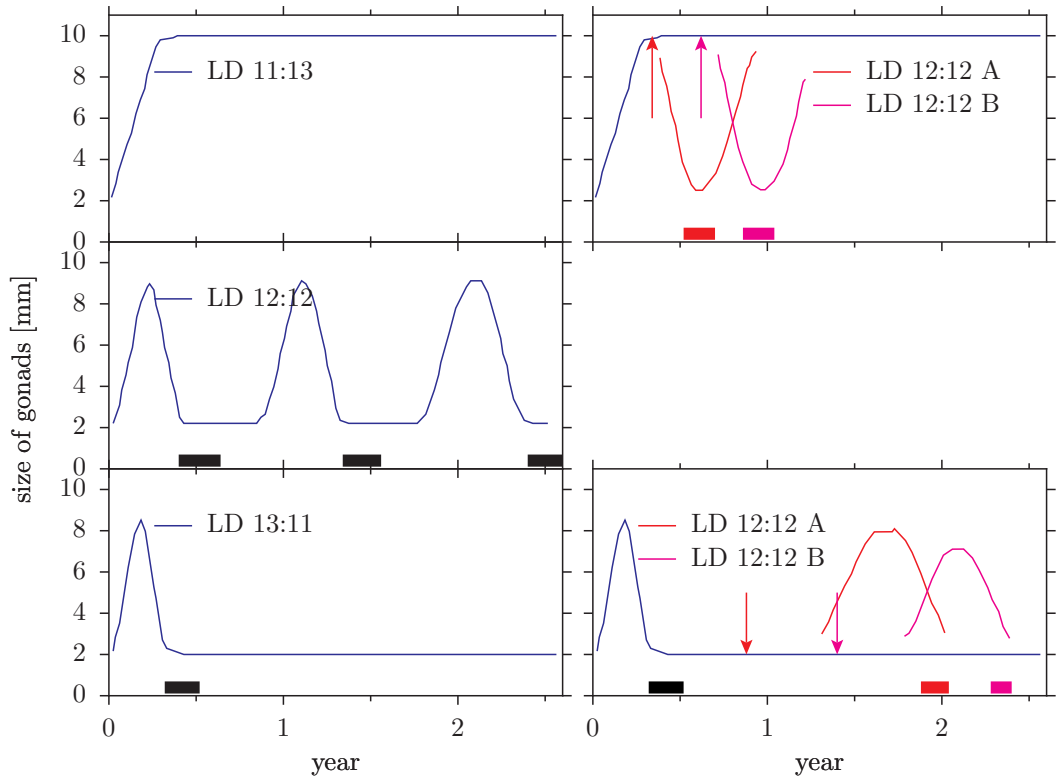

Figure 12.21: *Scheme of the annual rhythm of a starling under different light-dark-cycles of 11:13-, 12:12- and 13:11 hours. Under short day of 13:11 hours light-dark-cycle an annual rhythm is lacking, because the refractory state can be terminated by a long day only. Under a 13:11 hour light-dark-cycle the annual rhythm is arrested, because the long day prevents the birds from becoming refractory. Under a 12:12 hour light-dark-cycle, however, both, induction and termination of the refractory state are able to occur. If the birds are transferred from a 11:13 hour light-dark-cycle into a 12:12 hour light-dark-cycle (at two different times: begin of red curve, with text top right) or from a 13:11 hour light-dark-cycle (at two different times: begin of red curve, with text bottom right)) into a 12:12 hour light-dark-cycle, the gonads develop and molt begins after a certain time in respect to the transfer into the 12:12 hour light-dark-cycle (red curve for gonad size and bars for molt). Each January marked. After [543]*

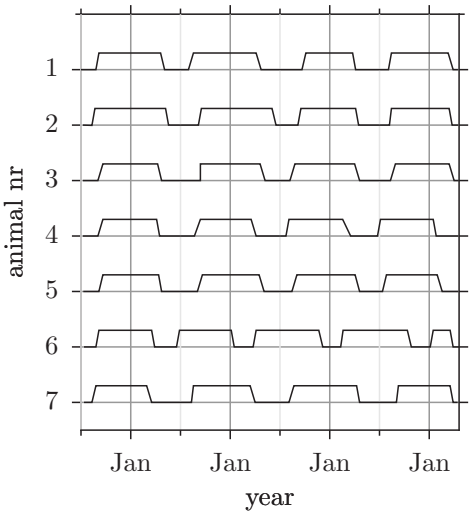

Figure 12.22: *Ground squirrels (*Spermo/-philus lateralis*, seven shown) were kept for four years at 3^0C under a 12:12 hour light-dark-cycle. The bars indicate the time of hibernation of the individual animals. The mean period length is 11 instead of 12 months, which signifies an endogenous annual rhythm. After [1148]*

less constant temperature. An endogenous annual rhythm makes sure that the right season is not missed and that hibernation is terminated in time. But even under constant conditions of an air conditioned room they continue to show hibernation. However, the period length deviates from the length of a year.[2] We are thus dealing with an *endogenous* annual rhythm (figure 12.22).

But even if time cues are perceived (photoperiodism, environmental temperature, food, light) and an endogenous annual rhythm is not needed, an internal annual clock could serve as a buffer system between environment and physiology of the organism. It protects against environmental disturbances such as weather and puts a certain inertia into it.

But not in all cases hibernation is controlled by an endogenous annual rhythm. Therefore it is distinguished between *permissive* and *obligate* hibernation.

Why hibernation?

Originally mammals were small insectivors with a nocturnal habit. The body temperature was around 30^0C, metabolism corresponded to that of reptiles (1/4 of recent mammals). Higher body temperatures were not tolerable: Because of the small size the water loss would have been too risky. Mind you that the body can be cooled only by transpiration, if its temperature is higher than that of the environment. With increasing size, however, body temperature could be kept at a higher level. In this way the mammals were living *internally in the tropics*. Afterward *homeothermia* was 'invented'. It allowed mammals, to be active also during the day at high environmental temperatures and thus to acclimatize to all climatic conditions of the earth.

Homeothermia has a high selective advantage: Faster reactivity, even during the night (at which the body temperature of poikilotherms decreases to the temperature of the environment), the ability to survive under extreme conditions. Even under high environmental temperatures the body can now be cooled, thus avoiding lethal temperatures (at 45^0C proteins coagulate).

During the winter larger mammals increase their thermal insulation (fat, winter fur) and are thus able to withstand low

[2] It might, for instance, be 300 days only instead of 365 days. Of 61 animals only 13 exhibited an annual rhythm with periods exceeding 365 d. The longest period was 445 days, the shortest 229 days.

temperatures much better. Often they seek protection in burrows and collect food reserves. In small mammals such as rodents, however, the costs might be too high. They possess an unfavorable relation between body volume and body surface. This implies a high metabolism at low environmental temperatures (in mice, metabolism is about 20 times higher as in sheep). Small mammals[3] do indeed adapt to the winter by increasing metabolism. Others however terminate the homeothermic state (about 37^0) during the winter and begin to hibernate. The body temperature drops to or close to environmental temperatures. 0^0C is the lowest limit. If this limit is reached, the homeothermic control takes place again for some time. Mammals during hibernation can be termed *heterothermic* animals, since they are in this state between poikilotherms and homeothermic animals by using for their body temperature a summer and a winter program.

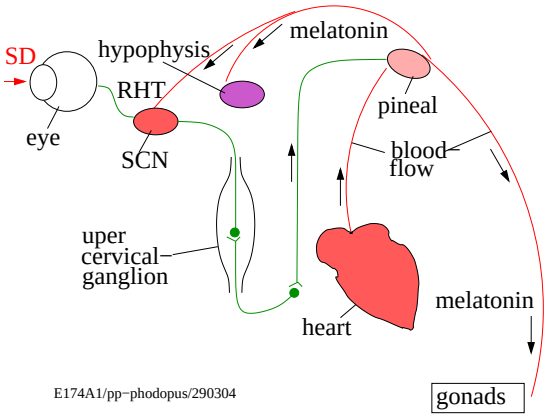

Figure 12.23: *Photoperiodic induction of hibernation in* Phodopus*: The retina perceives photoperiodically active light of short days (SD, red) and transfers the signal via the retinohypothalamic tract RHT to the suprachiasmatic nucleus SCN (details: figure 3.16). From there the signals pass the upper cervical ganglion (green) and reach the pineal organ (pineal, pink). The pineal is induced to secret more melatonin as compared to long days (into the blood stream). After onset of hibernation the melatonin concentration increases strongly and the normally observed variations between day and night (1:20) disappear. Melatonin is thus a signal for short day. These thermotropic reactions occur only in late summer and fall*

External factors

Onset of hibernation might occur at all seasons, but in the spring the tendency for hibernation is much lower. Usually it is induced by short days and low external temperatures in the fall. Before hibernation begins, more and more phases of cold lethargy occur. It is controlled by an endogenous annual rhythm. Fat reserves are deposited.[4] The most important external factor for induction of hibernation is photoperiod. The photoperiodic signals of the short day are perceived by the retina and transferred to the suprachiasmatic nucleus via the retinohypothalamic tract. From here the signal reaches via the cervical ganglion the pineal. It increases the secretion of melatonin (studies in the Siberian hamster *Phodopus*, figure 12.23). During hibernation the melatonin fluctuations are absent. They amount normally to

[3] they have to be heavier than 2.5 g, since otherwise not enough food can be gathered for the extremely high metabolism

[4] The amount of brown fat under short day conditions is increased in hamsters and so is the frequency of mitosis. Brown fat improves the heat production. The mitochondria possess a protein, which increases the conductivity of the mitochondrial membranes and uncouples respiration. Energy is in this way converted into heat instead of ATP.

a ratio of 1:20 between day and night. After the onset of hibernation the melatonin concentration increases rapidly. Melatonin promotes also cold resistance. The gonads retard. Melatonin is thus a signal for short day. The thermotropic reactions are limited to the late summer and fall.

Other external factors influence hibernation: It is more pronounced in animals further in the north. In these northern populations a timely (but not too early) termination of hibernation is important, in order to start reproduction rapidly, but late enough to avoid unfavorable conditions. A flexible time point would be averse; it has to be precise. In dry areas the environmental conditions are less precise and here flexibility is more important. The hibernation periods of typical hibernators are longer and less frequently interrupted.

The external temperature is important for hibernation to occur. The garden door mouse (*Eliomys quercinus*) kept in a 12:12 hour light-dark-cycle at 12^0 enters hibernation. But at 22^0 no hibernation occurs.

After having entered the hibernation state the hibernator does not stay continuously in this state. Instead an internal oscillator brings the animal back to its euthermic life for usually less than 24 hours approximately every 14 days ([456], [59], see figure 12.24). Torpor duration between the wake-up bouts depends on the ambient temperature and is shorter at higher temperatures. Timing of an arousal seems to be determined by the ambient temperature or the rate of metabolism during the preceding hibernation bout. The energetic costs of these arousals are high. Different explanations have been proposed why hibernators awake. According to a recent hypothesis ([301]) the hibernators are increasingly slow-wave-sleep deprived during hibernation and have to restore this deprivation with euthermic non-REM sleep. According to the two-process model of sleep regulation (see figure 2.8) process S would increase continuously during hibernation, but with a reduced rate, until it finally reaches a threshold at which arousal is triggered. [570], however, stress similarities between hibernation and sleep.

Hibernation, torpor and circadian clock

It was recently found that torpor does not only serve as a means to survive conditions with low environmental temperatures and shortage in food supply, but is also found in birds, marsupials and mammals under otherwise favorable conditions as a way to save energy ([595]). This torpor can cover whole seasons of several months, but might occur also on a daily basis.

Hibernation (actually aestivation, since it occurs during the dry season) combined with a daily torpor was found in *Cheirogaleus medius*, a lemur from Madagascar. It hibernates in tree holes. The course of its body temperature and oxygen consumption were telemetrically recorded in a field study by [292]. As shown in figure 12.25, the drop in body temperature occurs during the second part of the night and stays low for about 10 hours until the early morning. Oxygen consumption as a measure of the metabolic rate reflects this pattern. Outside temperature and less so tree-hole temperature drop several hours earlier as compared to the body temperature. Despite the relatively high body temperatures during early night the animals never arouse completely during the hibernation season. This is in contrast to hibernators with low body temperature throughout most of the hibernation period: They have to arouse periodically (see figure 12.24). Energy savings probably exceed those of animals which undergo a

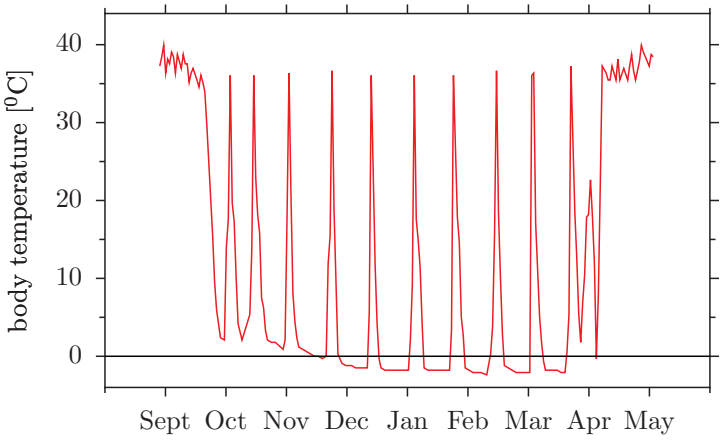

Figure 12.24: *Seasonal telemetric record of body temperature of an Arctic ground squirrel (*Spermophilus parryi*) throughout its hibernation season on the north slope of a mountain in Alaska. After [140]*

daily torpor without hibernating.

It is not known whether the torpor found in these lemurs occurs also under constant temperature conditions and whether an annual rhythm underlies the hibernation.

The results of laboratory studies on hibernation do often not correspond to the behavior in free-ranging animals. Telemetrically transferred data from temperature sensors showed that torpor in the field is more frequent, deeper and longer ([482]). Circadian rhythms of body temperature are found in hibernating laboratory animals, but not in field animals, which are in constant dark and under very quiet conditions. Either the body temperature is not coupled to the circadian pacemaker, or the pacemaker is non-functional in hibernating animals in the field ([436]).

The temporal pattern of the wake-up bouts during hibernation at prolonged 6^0C were studied in the homozygous (-/-) and heterozygous (+/-) tau mutant of Syrian hamsters and compared to the +/+ genotypes. Although the period length of the circadian activity rhythm differs in the non hibernating animals (20hrs, 22 hrs and 24 hrs, respectively), wake-up bout duration during hibernation was statistically indistinguishable (88.8, 94.2 and 86.9 hrs). The circadian clock which controls locomotor activity does not seem to control the wake-up bout periodicity, since in this case the durations should differ ([1106]). There might, of course, be a separate circadian oscillator controlling the wake-up bouts, which was not affected by the mutation.

Physiology of hibernation

Homeothermic animals keep their body temperature quite constant; in mammals this is usually about 37^0C. Temperature receptors in the hypothalamus make sure that the temperature is regulated as soon as it has deviated from the reference value by more than 0.5^0. The control center of the body temperature is located in the preoptic hypothalamic area (POAH). Furthermore peripheric control centers exist. This regulation occurs also during hibernation and during hypothermia, but at a lower

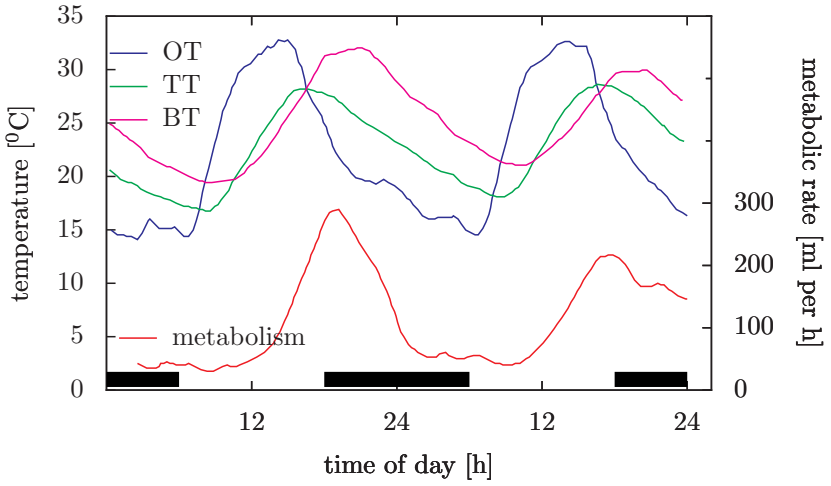

Figure 12.25: *Daily torpor of* Cheirogaleus medius, *a lemur from Madagascar. Body temperature (magenta) and oxygen consumption (red, right y-axis) were telemetrically recorded in a field study. In addition outside temperature (blue) and tree-hole temperature (green) are shown together with the daily light-dark cycle (black bars). After [292]*

reference value.

During hibernation the body temperature drops ($4 - 20^0$), water loss is low and metabolism amounts to 10 - 15 % of the normal value only. At a body temperature of 4^0 the O_2 consumption drops to 1/15 - 1/30 of the normal value. Respiration frequency and heart rate drop too.

Hibernation is, however, not merely a state of reduced activity and metabolism. Changes between active and inactive patterns in several cell and tissue constituents were found ([1602]).

The organism is using now a saving program. In contrast to hypothermia in which the lowered body temperature can be increased again to normal values by external warming up only, the body temperature during hibernation continues to be under precise physiological control, although at a lower reference value. Hibernation is not a continuous event. Heat and cold receptors in the skin report constantly the external temperature to the hypothalamus.

Changes in the central nervous system, in the endocrine system, in the physiology and in the metabolism occur during hibernation. Memory is, however, not much influenced during hibernation: The animals remember for instance the location of old burrows after hibernation.

The intermediary metabolism changes during hibernation. Fat serves as a reserve and energy source. Brown fat is a heat source. In the later part of waking up periods glycogen is used instead of fat. White adipose tissue is an important secretory and endocrine organ. It secretes among others leptin, a critical signal for the control of energy balance and other processes. It might play a role also during cold adaptation and hibernation ([1486]).

The acid-base-conditions are also changed during hibernation. The carbon dioxide of respiration decreases the pH. This changes the activities of enzymes.[5] Hibernators exhibit bradycardia similar to the one seen in marine mammals during diving.

[5] For instance, the activity of the phosphofructokinase drops by 80%, if the pH decreases by 0.1 units.

DNA synthesis is extremely low during hibernation, but not completely inhibited. Red blood cells mature even at 8^{o}C. The cell division cycle is interrupted in G2 and M (also in G1?). Especially against the end of hibernation, when the animals wake up more frequently, the cells start dividing again and are replaced. The eyes show during hibernation more pronounced degrading processes of the visual cells. After hibernation they are repaired ([1221]).

Electrical activity of the brain during hibernation is only 26% of the normal value. As compared to the normal sleep the EEG is flatter and much slower during hibernation. The sleeping pattern is completely changed. During deep hibernation the electrical activity of the brain has come to a complete rest and the cerebral cortex is silent during hibernation in the Syrian hamster, but not in the marmots. The vegetative nerve system is involved during the frequent wake up periods. The parasympathetic nervous system is inactive during hibernation. It becomes active after wake-up only. The Syrian hamster wakes up easily, the Turkish hamster not.

The SCN plays a prominent role in initiating and terminating hibernation. The neuropeptides and neurotransmitters transmitting light signals to the SCN (glutamate, pituitary-adenylyl cyclase activating peptide, neuropeptide Y were almost inactive during hibernation. However, signals to the SCN from other parts within the brain (serotonin, substance P, somatostatin, enkephalin) remained active throughout hibernation ([1099]).

The immune reactions are weaker during hibernation. Sick animals do not enter hibernation. They die when body temperature drops.

Hibernating animals wake up if disturbed but also spontaneously. The reasons for spontaneous arousal are unknown. It is discussed, that too many deposition products have accumulated, that the blood sugar level is too low, that an endogenous rhythm is responsible for it.

Gonads do not develop during hibernation.

Male ground squirrel arouse spontaneously from hibernation, females and young animals later, when food is available again.

Pinealectomy prevents hibernation. If the SCN is destroyed, the annual rhythms of reproduction and of body weight dissociate from each other. This speaks in favor of several circannual rhythms. Which of these circannual rhythms is responsible for hibernation is unknown. The effect of complete SCN ablation on the hibernation rhythms of ground squirrels were studied by [1273] under conditions of low temperature (6.5^{0}C) during 5 to 7 years.

Recently gene expression and protein adaptations have been compared between hibernating and non-hibernating species and in animals in the hibernating and non-hibernating state ([1426], [959]).

Theories of hibernation

Seasonal hibernation is a phenomenon which is still poorly understood. How induction, maintenance and arousal is controlled is mainly unknown. Different theories have been proposed which explain hibernation (see [595], [483], [199], [612], [939], [1528]), among them:

1. According to [1494] hibernation and arousal from hibernation are independent of the thermogenesis-regulation. Instead, the parasympathetic-sympathetic activity controls hibernation. There is an autonomous reaction to maintain hibernation. The parasympathetic system controls the onset of hibernation and the later part of arousal. Experiments with infusions of drugs into the blood or into specific brain areas speak in favor of this hypothesis.
   However the parasympathetic influences seem to be only fine controls, not part of basic processes of hibernation. Once

hibernation has started, the parasympathicus has lost its influence. Temperature effects are to be expected after changes in metabolism only.

2. According to [611] hibernation is a prolonged slow wave sleep. This part of the sleep is characterized by a high amount of metabolic saving.
   However, according to newer studies, the animals are not sleeping during hibernation. After arousal they possess a high sleep deficit, and timing of arousal might be a function of accumulating sleep debt ([1485], [301]).

3. According to [297] a hibernation-trigger-substance exists: If blood of hibernating animals is repeatedly injected into ground squirrel at normal temperature, hibernation is induced. A dialysate through membranes, which do not pass molecules above 5000 Da, lead still to hibernation. The substance is not species-specific. Even in animals, which do not hibernate such as rhesus monkeys and macaque, the heart frequency is lowered. The residue does not induce hibernation. It was tried to characterize this trigger-substance. The brown fat as a 'hibernating gland' was excluded. Pancreas, insulin, adrenal gland, corticotropic hormone, brain extracts, electrolytes are likewise no hibernation-trigger substances. It is unknown, whether the trigger-substance is a hormone. Do anti-trigger-substances exist?

4. Kondo and coworkers ([805], [804], [1362]) studied blood proteins, which are specific for hibernation of mammals. In the squirrel *Tamias asiaticus* 4 HP (=hibernation specific proteins) proteins were found in the blood plasma and their transcripts, which show a low concentration during hibernation. This was observed also during free run at 4^0C in continuous darkness for more than 5 years. The period length amounted to 8-12 months. It is thus an endogenous annual rhythm. It was found also in the mRNA. This rhythm was also found in animals, which were kept under a 12:12 hour light-dark-cycle at 23^0C. Under these conditions normally hibernation does not take place. The proteins HP 20, 25 and 27 were also found in other hibernating rodents, but not in the non-hibernating rat. Thyroxine may be involved in the regulation of HP during hibernation

5. According to Beckman and coworkers ([74], [75]) the reticular formation of the mid-brain (MRF) and of the preoptic anterior hypothalamus (POAH) are a mechanism, which facilitate neuronal inputs into the hypothalamus and the hippocampus.

12.4.5 Properties of annual rhythms

The mere occurrance of annual rhythms does not necessarily imply an underlying endogenous annual clockwork. Hidden Zeitgeber could induce the rhythm exogenously. A number of **criteria** must be fulfilled, before we are allowed to speak of a circannual rhythm:

1. The periodicity has to be exhibited during a number of cycles.

2. The period length should deviate from exactly 12 months, being either somewhat shorter or longer.

3. This endogenous annual rhythm must be entrainable to exactly 12 months by annual Zeitgeber.

4. A true endogenous annual rhythm should be robust and more or less temperature-compensated.

Annual rhythms possess the following **properties**:

1. They occur under constant conditions (this must not necessarily be continuous light or continuous darkness, but could for instance be a light-dark-cycle of 12:12 hours given all the time).

2. The period length is more or less independent of the length of the light period.

3. The period length deviates usually from 12 months, being usually shorter than a year[6].

4. The period length is independent of temperature (which is kept constant, but might for instance be 15^0 in one case, and 25^0C in another case).

5. Annual rhythms are inherited.

6. The range of entrainment is limited.

Some remarks:

Prerequisites for testing for circannual rhythms are constant conditions, which are however seldom found in nature (such as in certain parts of the tropics), but can be produced in air conditioned chambers. Temperature and light conditions (especially photoperiod) must be constant.

[6] If length is exactly 12 months, a hidden Zeitgeber might have induced it.

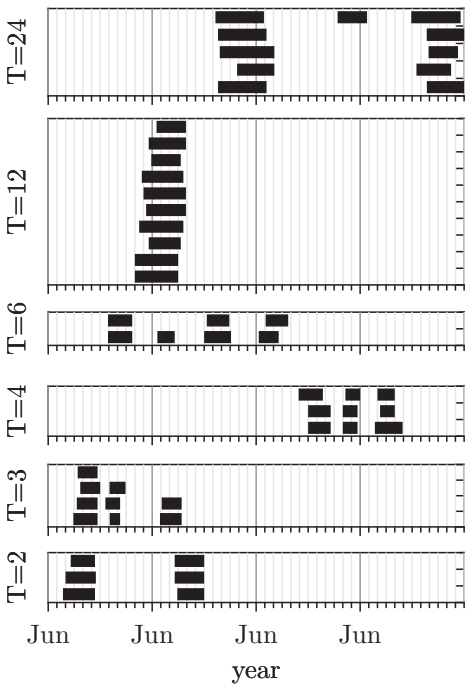

Figure 12.26: *Range of entrainment of the annual rhythm of antler growth (bars) in Sikka-deer* Cervus nippon *under artificial 'years' with sinusoidally modulated length of light period (from T=24 to T=2 month). After [515]*

The range of entrainment of the circannual system is limited, as is to be expected for all endogenous oscillators, but is quite large in comparison with circadian rhythms. In the annual growth rhythm of the antler of *Cervus nippon* for instance even 4 months, but also 24 months with sinusoidal change of the length of the light period can 'entrain' (figure 12.26).

The annual rhythms can be well expressed or less well, partly even in the same species and population. If the endogenous annual rhythm is less well expressed, the animals can react better to environmental factors. Life long circannual rhythms are found in the golden man-

tled ground squirrel *Spermophilus lateralis* (hibernation), the squirrel *Tamias striatus* (body weight), the garden warbler and black cap (molting). In other cases the annual rhythm is damped. This is the case in the annual rhythm of body weight in an American dickcissel (*Spiza americana*) and in the molting and migratory restlessness in *Phylloscopus collybita* and *Sylvia communis.*

12.4.6 Zeitgeber of the annual rhythms, synchronization

The most important Zeitgeber of circannual rhythms is normally photoperiod. The antler growth of *Cervus nippon* occurs from April to June, while light period increases ([516]). In *Sylvia borin* it was also shown that light period is a Zeitgeber of the annual rhythm. In figure 12.27 the natural annual rhythm of the estrus-activity in 'Southdown'-sheep is shown. It is during the winter (of the southern hemisphere, therefore May/June) highest. Using an artificial annual change of the photoperiod (shifted by 6 months against the natural one, and with a higher amplitude of the day-lengths) it can be dislocated. The highest values are now found during January.

Marmot (*Marmot monax*) were flown from a natural habitat in Pennsylvania (40^0N) to Sydney (34^0S) and kept there under natural conditions. Three years later their annual rhythm was shifted by 6 months (figure 12.28 and [294]). It is unknown, which Zeitgeber are responsible in this case for the synchronization. It is probably the day-length.

Other Zeitgeber can synchronize the annual rhythm. For instance food supply (nutrient deficiency, [1044]), monsoon (precipitation), social Zeitgeber ([1042]). But a change in temperature is also able to shift the annual rhythm ([1149], [1044]).

The photoreceptors for photoperiodic Zeitgeber of the annual rhythms are unknown.

12.4.7 Physiological basis and localization, hormones, models of circannual rhythms

Where are the generators for annual rhythms situated in the brain? According to [1422] and [834] the hypothalamus seems to be involved. Substrate of the neuroendocrine axis are the eye, the hypothalamus and the gonads.

Is an internal coincidence between neurotransmitter-rhythms the basis of circannual rhythms? And can circannual rhythm be reset by neurotransmitter? And how does the time measurement function?

Whether hormones are of significance for circannual rhythms can be studied by elimination or addition of hormones.

The gonads of birds and mammals do not seem to be components of the circannual rhythm, since castrated animals show still an annual rhythm. Onset and end of secretion of gonadotrophins by the hypophysis-hypothalamus-system is controlled by the circannual rhythm. But the rhythm functions independently of the secretion of the gonadal hormones. This was shown in females of ground squirrels ([1617]) and male starlings ([543]).

The pineal has hardly an influence on circannual rhythms. But the annual rhythm of pinealectomized starlings is clearer as compared to control animals. The pineal is however important for the photoperiodic effect. In mammals pinealectomy has also no effect on the circannual rhythm. Without pineal the annual rhythm is more pronounced,

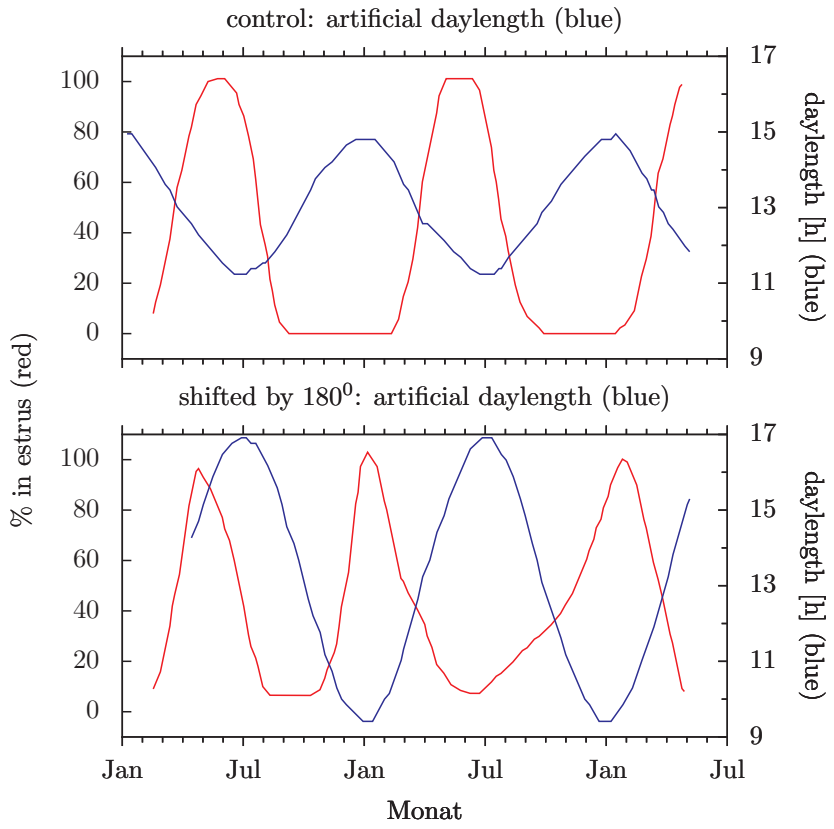

Figure 12.27: *The natural annual rhythm of estrus-activity in 'South/-down'-sheep is shown in the upper part of the figure (red curve) together with the changes in natural day-length for the geographical latitude of* $30^0 30'$ *South (blue curve, day-length changes between 9 and 17 hours). The highest estrus activity is found in the winter (of the southern hemisphere, therefore May/June). It can be shifted against the natural course by an artificial annual change of the photoperiod (bottom graph, red curve) by 6 months, that is* 180^0 *shifted in respect to the natural one in upper graph (day-length changes between 9 and 17 hours, blue curve in lower graph). The highest values of estrus activity are now found in January. After [1475]*

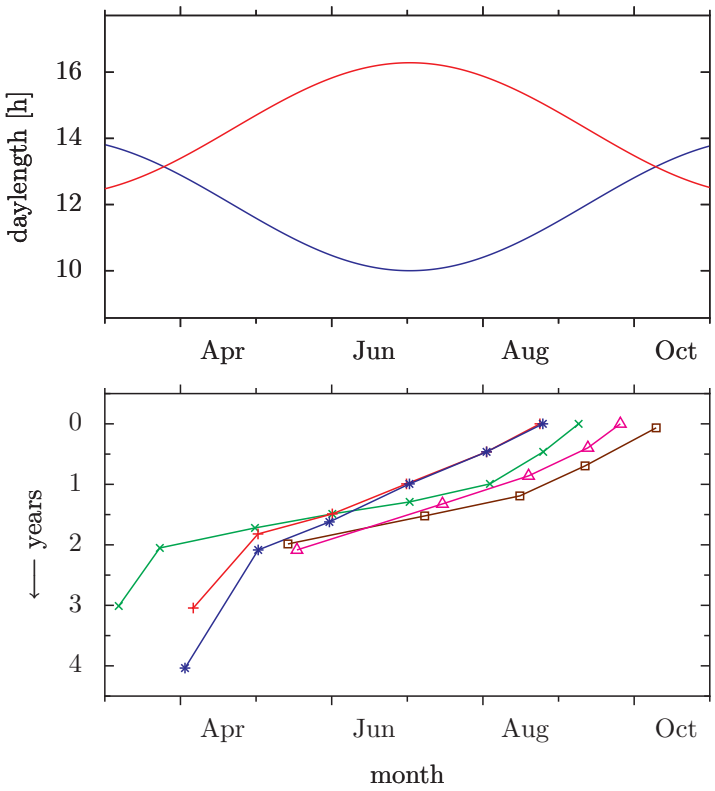

Figure 12.28: *Seven marmots (*Marmota monax*, different colors) were transferred from Pennsylvania (40^0N) to Sydney (34^0S) during the first year and kept there outdoors. The day-length for Pennsylvania and Sydney as a function of the time of the year is shown in the upper curve. Three years later the annual rhythm of the body weight of the animals was advanced by six months and showed now the same phase relationship to the seasons (and the day-length) as in Pennsylvania. After [294]*

as in starlings, the period length is somewhat longer in *Mustela*, somewhat shorter in ground squirrels as compared to intact animals. Pinealectomy has, however, a strong effect on the synchronization of the annual rhythm. In *Mustela* and in the sheep the estrus is strongly retarded under natural light-dark-cycles.

Neither the ventromedial hypothalamus nor the paraventral nucleus nor the central gray zone of the mid brain are responsible for the occurrence of annual rhythms. The SCN does influence under certain conditions the coupling between circannual rhythms and body weight and reproduction.

12.5 Genetics of annual rhythms

Annual rhythms are genetically determined: Offsprings of *Citellus lateralis*, born and kept under constant conditions possess an annual rhythm. Black caps (*Sylvia atricapilla*) breed in Europe once a year. The African strain of the Cap Verde islands breeds however twice per year. Crossing both strains lead to offsprings with an intermediary behavior of these different annual patterns (figure 12.29, [101]).

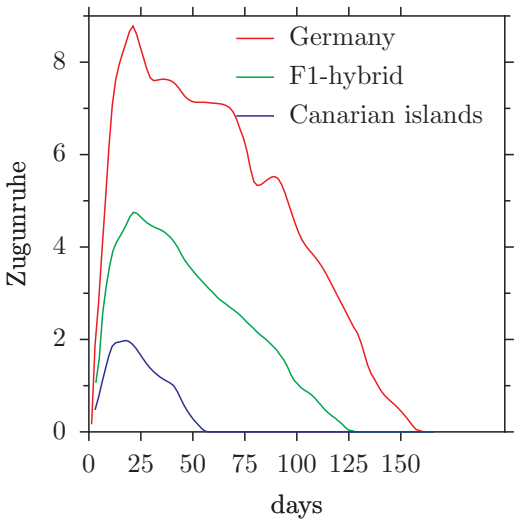

Figure 12.29: *Amount and duration of restlessness (number of nocturnal 0.5 hour intervals with activity) differ considerably between black caps (*Sylvia atricapilla*) from Germany (solid curve) and an African variety from the Canary islands (dotted curve). The offsprings (F1-hybrids, stippled curve) show an intermediary behavior. After [101]*

12.6 Adaptive significance and use of annual rhythms

Annual rhythms are wide spread among organisms. What is their significance? If annual Zeitgeber are missing as is the case in the tropics or at the bottom of the sea (see *Lingulodinium*), an annual clock has certainly its advantage. Furthermore organisms with an annual clock can protect

themselves better against unreliable informations of the environment such as temperature and humidity. Seasons can be anticipated, for instance in seeds of plants.

An internal annual calendar is also of advantage, if animals live under constant conditions, but have to initiate at certain times of the year certain events. This is the case for the termination of hibernation or for migration into the breeding quarters in migratory birds, which over-winter in the tropics. Without circannual clock these organisms would be the play of environmental conditions, and a temporary irregularity in the periodicity of the environment could have disastrous consequences. Instead, an internal calendar allows a timed behavior. The animals are not caught in surprise, but are prepared for the changes of the environment 'internally'. This is perhaps also the reason, why most of the endogenous annual rhythms are shorter than 12 months. The internal annual clock 'rings the bell' already before the expected event and the animals are able to prepare themselves for it (see discussion in [542]). In this way reproduction and hibernation occur during the right season, the sexes can synchronize to each other at the onset of the reproductive season, and specific timing programs can initiate sequences of events.

The circannual clock controls also duration and amplitude of events (figure 12.30). In the case shown in the figure the amount of fat deposited for the different phases of hibernation is pre-programmed. In six different warbler-species (Sylvia borin, cantillans, communis, atricapillata, melanocephala and sarda) the duration, amount and the timing pattern of migratory restlessness is pre-programmed. Using vector-navigation even the inexperienced migratory bird finds its target automatically.

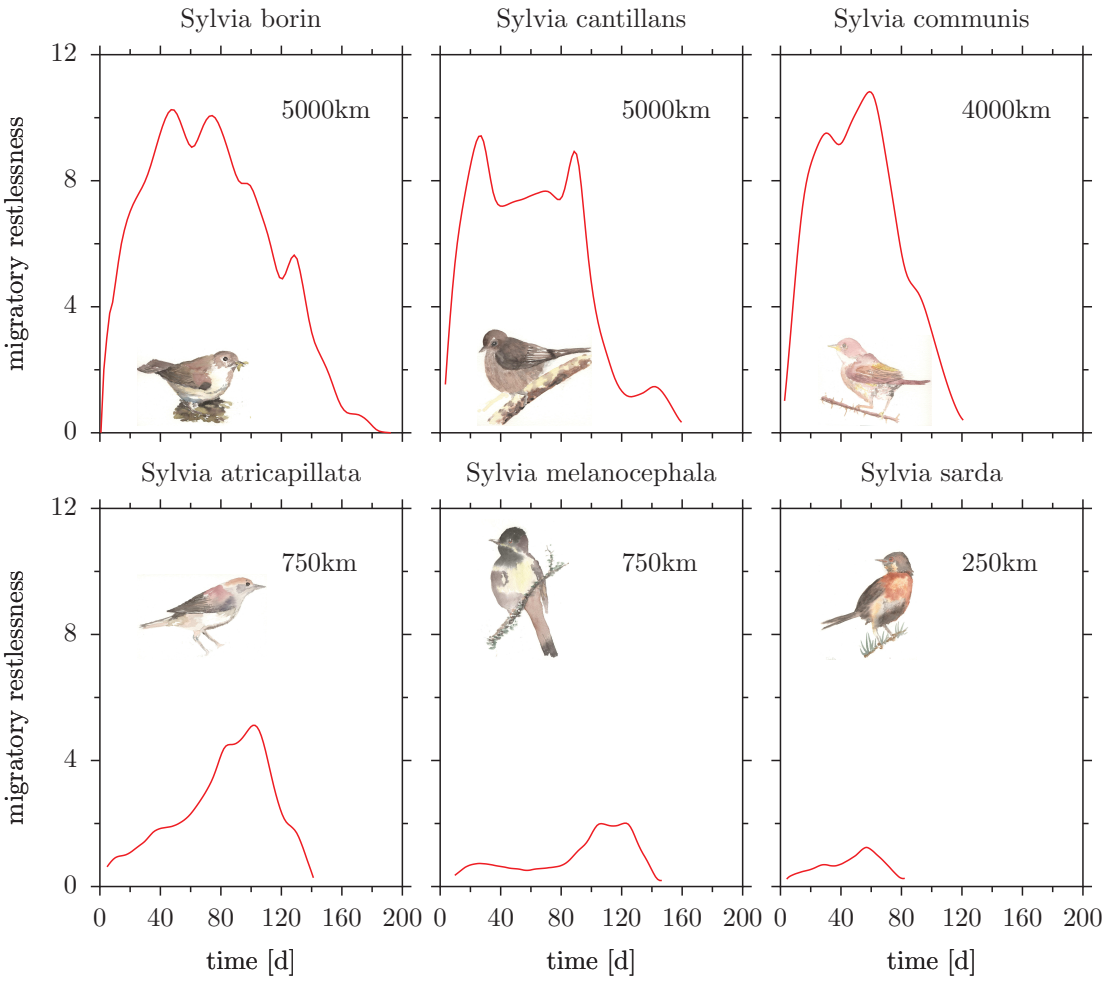

Figure 12.30: *An annual clock controls duration and amplitude of events. In the* Sylvia-*species* borin, cantillans, communis, atricapillata, melanocephala *and* sarda *the duration, amount and time pattern of migratory restlessness is pre-programmed. The nocturnal activity (number of nocturnal 0.5 hour-intervals with activity) is the highest and most extended in species with long distance migration. After [97]*

13 Photoperiodism

To synchronize the annual rhythm with the seasons, Zeitgeber of the environment must be perceived and they must affect the annual rhythm. The most reliable time cue for the seasons is photoperiod. Therefore photoperiodic informations are used by numerous organisms to synchronize their annual oscillator with the environment. There are, on the other hand, also quite a number of organisms, which exhibit photoperiodic reactions without possessing an annual rhythm. In the following examples are presented for photoperiodic reactions. The first one is a unicellular, Lingulodinium. *Afterward photoperiodic reactions of plants are dealt with (storage organs, seeds, flower induction). The diapause of insects, a resting stage, is often photoperiodically controlled. Furthermore, the reproduction of mammals is in most cases under the control of the day-length. As an example for the photoperiodism in birds the control of reproduction in quails is given.*

13.1 Photoperiodism in an alga

We got to know already the dinoflagellate *Lingulodinium polyedrum*: It exhibits a circadian rhythm of bioluminescence. In order to over-winter, this alga forms a cyst. It sinks to the bottom of the sea, casts its armored shell and forms a new encystment (cyst). Encystment of *Lingulodinium polyedrum* is photoperiodically controlled ([48]). At a water temperature below 16^0C these algae develop under short day conditions asexual cysts as a resting stage. Figure 13.1 shows the percentage of cysts

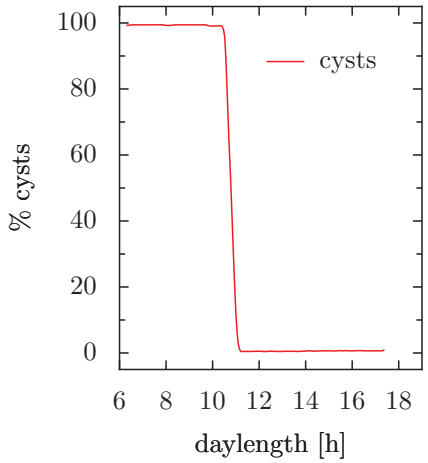

Figure 13.1: *In short day (up to a day-length of 10.5 hours)* Lingulodinium polyedrum *forms cysts. No cysts are formed if the daily light period is 11 hours or longer. The percentage of cysts formed is plotted as a function of day-length. After [48]*

in the population as function of the length of the light period. At 11 hours light per day no cysts are found, at 10.5 hours light all cells are encysted. It is known from photoperiodic reactions that a short day effect can be canceled by light in the middle of the dark period and a long day reaction takes place instead. The same here: The cyst formation can be canceled by a two hour light pulse during the middle of the dark period. This indicates that we are dealing with a true photoperiodic reaction and not a reaction, which depends on the amount of light received.

For the photoperiodic reaction to occur, the temperature of the water has, however, to be 16^0C or lower (upper part of figure 13.2, green curve under short day conditions of 10:14 hours light dark-cycles, compare with non-inductive conditions of 11:13 hours light-dark-cycles, blue curve). At higher temperatures no cysts are formed in spite of short day (12:12 hours light dark-cycles, green curve).

But asexual cysts are also formed under long day conditions, if melatonin or even more so the melatonin-analogue 5-methoxytryptamin is added (figure 13.2, left part, red curve, [49]). With 5-Methoxytryptamin cyst formation can be induced even in continuous light at 20^0C.

In vertebrates photoperiodic signals of the environment are also transmitted by melatonin (in the pineal) (see 20.8). Melatonin reaches in *Lingulodinium* a higher concentration as in the pineal. As in vertebrates the melatonin concentration fluctuates also in *Lingulodinium* in a diurnal and a circadian way ([51], figure 13.3). In both cases the maximum of the synthesis is shortly after the onset of the dark period ([1169]).

Melatonin is an indolamin and widely distributed among animals. It is a radicals

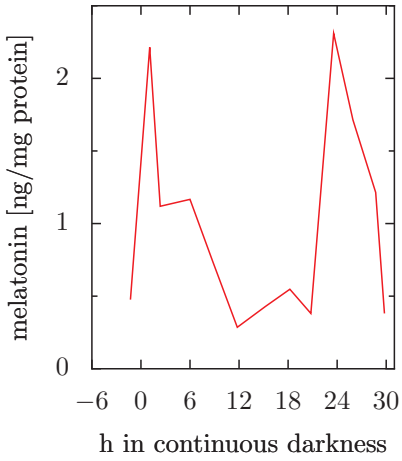

Figure 13.3: *Circadian rhythm of melatonin (ng/mg protein) in* Lingulodinium polyedrum *after transfer of the culture from a 12:12 hour light-dark-cycle in continuous darkness (at time 0, abscissa). After [51]*

seizer: Superoxide anions formed by light are broken down by melatonin with hemin as a catalyst to form kynuramin (AFMK) ([564]). Whereas melatonin serves the organisms as a signal of darkness, AFMK is an indicator of light. The events seem to be quite similar in unicellular and in mammals and point to a common origin during evolution.

Photoperiodic reactions are known also from other algae (Conchiospores of *Ulva*, monospore formation in *Porphyra*, see [1227], [321], overview [933] and [335])

13.2 Photoperiodism in plants

In many plants certain developmental steps and physiological events are under photoperiodic control. In this section some examples are given. First photoperiodic reactions of tuber- and bulb formation are discussed. Afterward the induction of

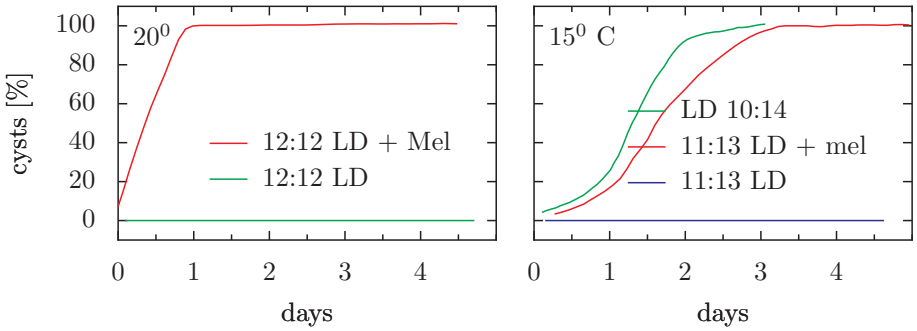

Figure 13.2: *In shortdays of 10:14 hours light-dark-cycles (right part, green curve) cysts are formed after a few days (percentage, ordinate), whereas in longdays of 11:13 no cycsts are found (right part, blue curve). At temperatures beyond* 15^0C *no cysts are formed in* Lingulodinium polyedrum *in spite of short day (left part, 12:12 light-dark cycles, green curve). If methoxytryptamin* $(2 * 10^{-5}M)$ *is added to the medium (left part, red curve), cysts are formed in the next days (abscissa) in spite of long day conditions (red curve in right part) and even at higher temperatures (red curve in left part). After [49]*

seed rest and seed germination by the photoperiod is presented. Finally some examples for photoperiodic flower induction are given.

A photoperiodic reaction can be induced by short day or by long day. Important is not the absolute length of the light period (or dark period). Decisive for the photoperiodic reaction is, whether a critical length is exceeded or falls short (figure 13.11). Furthermore, during the development of a plant photoperiodic decisions can be met several times, and they might belong to different types (long day, short day, example: *Zea mays*).

A detailed older presentation of photoperiodism in plants is by [1515]. A recent review is by [1469].

13.2.1 Photoperiodic control of storage organs.

Storage organs are formed by swelling of various tissue such as stalks (tubers and rhizoma), roots (leads to tuberous roots and root tubers), leaves (breading tubers). With the help of these storage organs plants can survive unfavorable conditions and store material (table 13.1). As in photoperiodic flower induction the recipient organ for the photoperiodic stimulus of storage organs is also the leaf. The dark period is responsible for the reaction. The induction occurs via the pigment system phytochrome (see page 437). After the induction the stimulus is transmitted to the target organ.

Table 13.1: *Storage organs of plants*

favoured by short days		
groundnut (*Apios tuberosa*)	root tubers	[479]
Begonia evansiana	aerial stem tubers	[404]
Begonia socotrana	aerial stem tubers	[1094]
Begonia tuberhybrida cv *Camelliaflora*	underground stem tuber	[888]
cv *Multiflora*	underground and aerial stem tuber	[888]
Dahlia hyprida	root tubers	[1094]
Discorea divaricata	aerial axillary stem tubers	[479]
yam (*Discorea alata*)	rot tubers	[479]
Gladiolus spec.	cormels	[29]
Helianthus tuberosus	underground stem tubers	[556]
potato (*Solanum tuberosum*)	underground stem tubers	[1530]
favoured by long days		
shallot (*Allium ascallonicum*)	bulbs	[697]
onion (*Allium cepa*)	bulbs	[948]
garlic (*Allium proliferum sativum*)	undergound and aerial bulbs	[1213]
Brodiaea laxa	corms	[447]

In *Begonia evansiana* one or two short days are already sufficient to induce the air tubers. In other cases such as bulbs of onions several photoperiodic cycles are needed and a photoperiodic counter is involved.

Here are some examples of photoperiodic control in storage organs:

Potato tubers

The tuber formation in potatoes is influenced by a number of factors, such as temperature, nitrogen content, physiological age of the plants and especially photoperiod. In the native potato species of southern America the tubers are induced in short day. Long day inhibits the tuber formation. The south American cultured varieties such as *Solanum demissum* and *Solanum tuberosum* ssp. *andigena* do also form tubers in short days only. In light periods beyond the critical day-length tuber formation is suppressed ([415]). Decisive is the length of the dark period. If night is interrupted by a light pulse, no tubers are formed. Red light is the most effective. Far red light cancels the red light effect. Phytochrome (see page 437) is therefore the photoreceptor for the photoperiodically effective light ([68]). It is the phytochrome B, as shown in experiments using antisens-plants (see [691]). Most European and north American cultivars show a weak photoperiodic reaction or they form tubers also in long days (early potatoes!).

The perception of the photoperiodically effective light is in the leaves. The length of the dark period is determined by a circadian timing system. If the dark period is longer than a critical duration, a signal is produced [1] and transmitted to the subterranean sprouts (stolons). The signal consists probably of an inducing substance, which accumulates under inductive conditions, and an inhibiting substance, which is diminished under inductive conditions. Gibberellins might serve as inhibiting substances ([1478]). Jasmonic acid and tuberonic acid are probably tuber promoting substances ([799], figure 13.4). Jasmonic acid is synthesized via lipoxygenase (LOX). The activity of LOX is increased by short day. It is furthermore known, that the synthesis of gib-

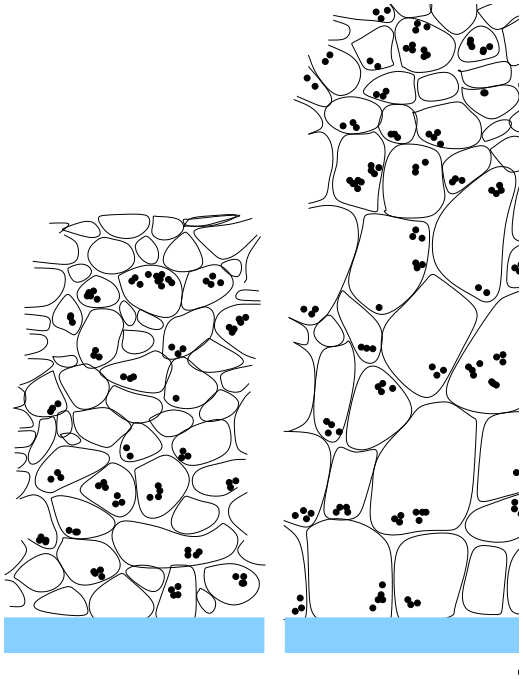

Figure 13.4: *If potato disks are put on agar, to which* 10^{-4}*M jasmonic acid was added, the cells expand (right part of the figure). Control without jasmonate (left). After [1452]*

[1] This signal is established by grafting. Induced leaves of the potato induce plants kept in long days to form tubers if grafting was successful. Leaves of tobacco plants induced in short days are also able to induce tuber formation in potatoes in longday conditions after grafting ([222], [957]).

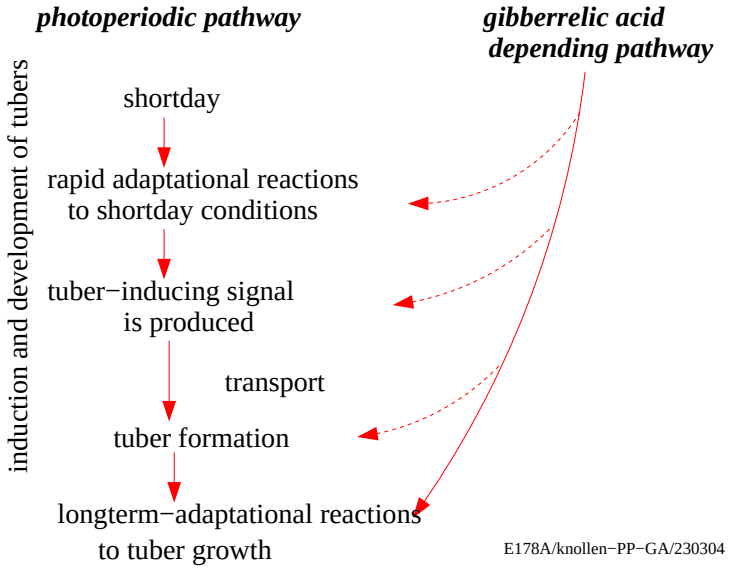

Figure 13.5: *How the development of tubers is controlled by a photoperiodic pathway (red arrows left) and how gibberrelic acid affects in special stages the photoperiodic pathway by inhibiting it (red arrows right)*

berellic acid is photoperiodically influenced ([500], figure 13.5). .

Potato tubers are formed by thickening of the stolons at their tips (figure 13.6). The cells expand perpendicular to the longitudinal axis and deposit starch.[2] The small scaly leaves at the potato tubers are shed early. The scars are still visible on the tuber. Axle buds are found here ('eyes'). They form later lateral shoots.

Bulbs

Bulbs are in contrast to tubers of potatoes mostly formed under long day conditions. The onions of *Allium cepa* and of garlic *Allium proliferum* are examples (figure 13.7). The yellow Zittau onion needs at least 14 hours long day. In more southern latitudes varieties are common such as the sweet Spanish onion, which forms bulbs also under shorter day-lengths (12-13 hours). The critical day-length is thus shorter. To form bulbs, at least 7 to 28 long days are necessary in *Allium ascalonicum* ([405]).

[2] Methyljasmonate inhibits longitudinal growth and promotes expansion in the radial direction.

13.2.2 Photoperiodic control of succulence

In numerous succulents the succulence of the leaves is photoperiodically controlled. In *Kalanchoe blossfeldiana*, for instance, small, rigid, succulent leaves are formed under short days. Under long days, however, the leaves are thin, flexible and large (figure 13.8). Even outgrown leaves become under short day conditions succulent and are three times thicker as compared to the long day leaves. The leaves become succulent by expansion of the cells perpendicular to the lamina and by water uptake (figure 13.9). The stimulus produced under short day conditions

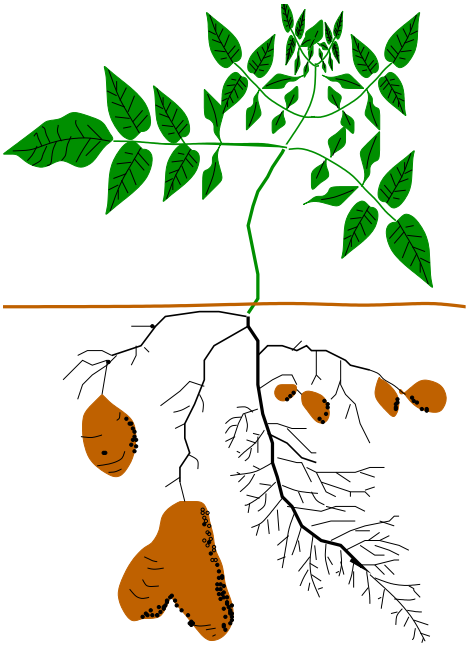

Figure 13.6: *Under short day tubers are formed at the end of the subterranean stolons of potato plants, which swell perpendicular to the longitudinal axis. The horizontal line represents the surface of the soil. The root system is below the stolons*

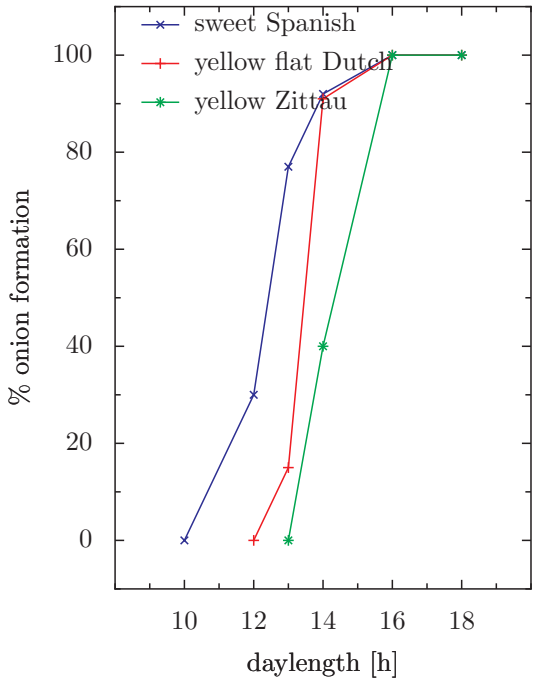

Figure 13.7: *Dependency of bulb formation (Percentage) in three different* Allium cepa *cultivars on the day-length (hours). After [948]*

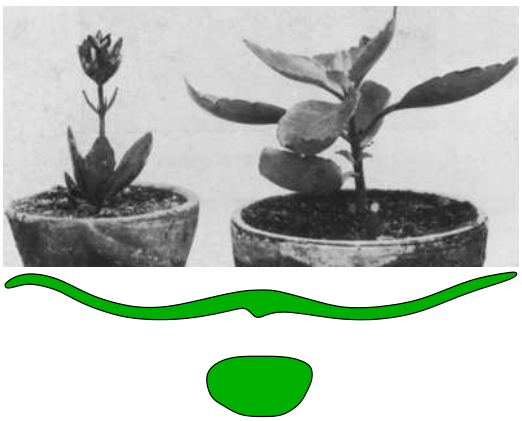

Figure 13.8: *leaves of* Kalanchoe blossfeldiana *in short day (left) and in long day (right). Below: Cross section through short day leaf (bottom) and a long day leaf (top). After [567]*

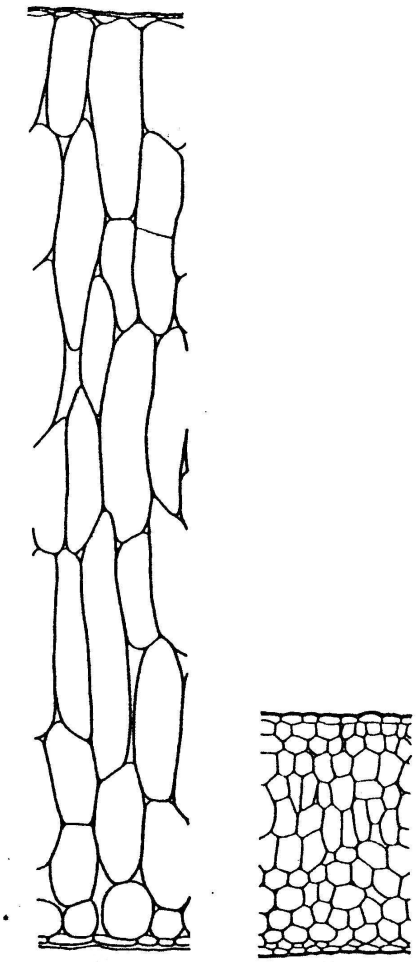

Figure 13.9: *Anatomy of leaves of* Kalanchoe blossfeldiana *in short day (left, K) and in long day (right, L) at the same magnification. After [567]*

can be grafted.

13.2.3 Other photoperiodic effects

Other photoperiodic effects are known in plants such as root formation, shedding of leaves, bud formation and bud dormancy in trees and bushes, stem elongation, vegetative growth, cambium activity, tissue differentiation, cold resistance, fertility, sex determination and -expression ([1515]).

13.2.4 Photoperiodic reactions in seed dormancy and seed germination

The development of an angiosperm plant was shown in figure 12.1. The embryo in the seed of a plant contains already all the tissue of the vegetative plant. Before it develops, it turns to a dormant stage. The seed is protected and can survive unfavorable environment conditions. This developmental arrest of development is not just the automatic consequence of embryogenesis, since exceptions are known (mangrove embryo). In many plants the seeds germinate, if water is available and thus gas exchange possible. In other cases additional photoperiodic signals or low temperature (vernalisation) is needed, to terminate the developmental arrest. Again in other cases the seed dormancy is controlled by an endogenous annual rhythm (section 12.1.1).

Examples for the photoperiodic control of seed germination are *Lactuca sativa*, *Betula pubescens* (figure 13.10) and *Betula pendula* ([1506]). Examples for the photoperiodic control of seed dormancy are *Desmodium barbatum*. Seeds which ripened on plants kept in short day (8 hour light period) have a higher germination rate as

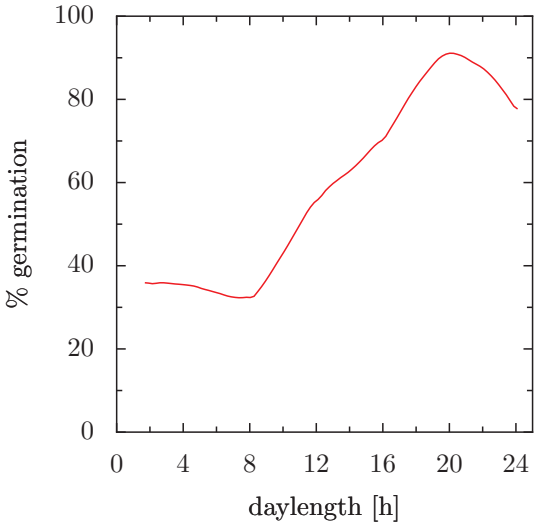

Figure 13.10: *The seeds of the birch tree* Betula pubescens *show under short day conditions (2 to 8 hours light per day) a low germination rate, whereas under long day (20:4 hour light-dark-cycle) 90 % germinate (15°C). After [106]*

seeds of plants which ripened in long days (18 hour light period) ([1391]).

13.2.5 Photoperiodism and flowering[3]

To flower and have seeds ripen to the right time of the year plants use a complicated control system. It activates the switch from the vegetative to the reproductive state of the apex. A part of this control system relies on the developmental state: The plants must have gone through a juvenile phase and must have reached a certain age before flowering occurs. This is the **autonomous way** of flower control. Another part of the control system receives environmental signals such as day-length, temperature and humidity of the soil. Plants do not flower before this **environment-reactive way** signals the right conditions. Whether the autonomous or the environment-reactive way predominates, depends on the plants and varies strongly ([38]). One of the environmental conditions which have to be met for flowering to occur is in many plants the day-length. Flowering is induced only after certain photoperiodic conditions are met. A recent book on photoperiodism is [1470].

In the flower induction photoperiodism was discovered by Garner and Allard who created also the term ([478]). Short day plants flower, if a critical light period is *subceeded*, long day day plants flower, if critical light period is *exceeded* (figure 13.11). For experiments the following plants are often used: The short day plants *Pharbitis nil* (morning glory), *Chenopodium rubrum* (red goose-foot), *Kalanchoe blossfeldiana*, *Glycine max* (soy bean), *Xanthium pennsylvanicum* , the long day plants *Lolium perenne* (darnel, a grass) and *Arabidopsis thaliana* (thale cress), to name a few (figure 13.12). Among the photoperiodically reacting plants are also some which need first short day and afterward long day in order to flower (so called short-long day plants). In the long-short day plants it is reversed ([411]). Day neutral plants flower independent of the day-length in short day and in long day conditions.

Photoperiodic flower induction is of paramount practical importance for agriculture and horticulture (review: [1301]).

The photoperiodic control of flower induction is a complex event and quite manifold. Studies of these processes are interesting and important, if the induction and development of flowers is the main aim. If one is, however, more interested in the

[3] for more recent results see http://w210.ub.uni-tuebingen.de/volltexte/2009/3762/

Figure 13.12: *Short day plants a:* Pharbitis nil *(morning glory), b:* Chenopodium rubrum *(red goos-foot), c:* Kalanchoe blossfeldiana, *d:* Glycine max *(soybean), and e:* Xanthium pennsylvanicum. *Long day plants f:* Lolium perenne *(darnel, a grass) and g:* Arabidopsis thaliana *(thale cress)*

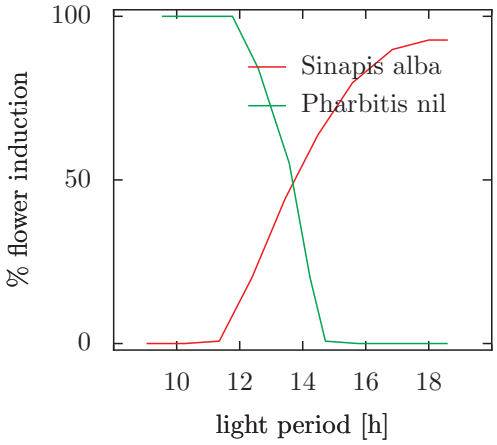

Figure 13.11: *Short day plants flower, if a critical light period is* subceeded, *long day plants, if a critical light period is* exceeded. *Green curve:* Pharbitis nil *strain Violet (short day plant). Red curve:* Sinapis alba *(long day plant). After [673] (*Pharbitis*) and after [94] (*Sinapis*)*

photoperiodic control of events, it is more advisable to use a more simple photoperiodically controlled process: For instance the switch from longitudinal to perpendicular growth or the induction of tuber formation.

From the photoperiodic stimulus to the flower formation: photoperiodic induction

The first step is the perception of the photoperiodic impulse. This occurs in the leaf. In the mesophyll and the epidermis of the leaf receptors are located for the photoperiodically acting light. They are different in the various plants. Phytochromes are the most common receptors in flower induction (see subsection 20.13.1). In *Crucifera* in addition to phytochrome blue light receptors are involved. A time measurement system and a photoperiodic counter play an important role (figure 13.13). Time is measured by a circadian system (see section 20.16). In short day plants a critical day-length (light period), in long day plants a critical dark period seems to be measured. Therefore the terms light-dominant and dark-dominant plants is also used. If a critical dark period is exceeded (short day plants) or subceeded (long day plants), substances are produced from the cells in the leaves, which are transported to the apex and switch it from vegetative to reproductive development. The plant is induced to flower.

Thus we are dealing with different events, which will be looked at more closely in the following, in order to understand flower induction:

1. In the leaf the photoperiodically active light is perceived by photoreceptors.

2. In the leaf the day-length is determined.

3. If day-length is adequate and enough inductive cycles have been presented, substances are produced in the leaf, which lead finally to flower induction.

4. These substances ('florigen') are transported to the apex.

5. Florigen switches the apex from vegetative growth to reproductive growth (flower induction).

6. In the switched apex changes of the gene activities occur which lead to flower formation.

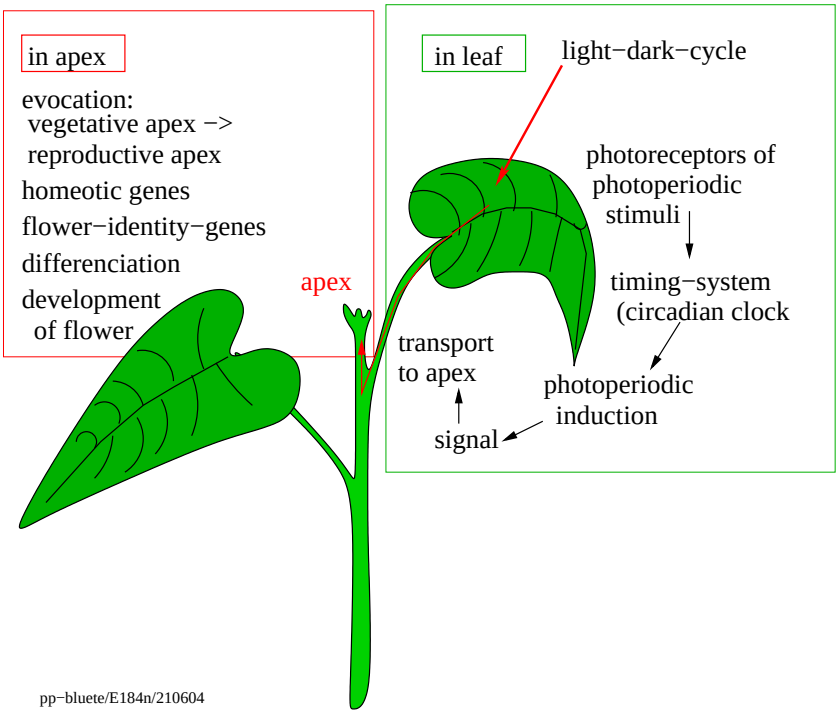

Figure 13.13: *In the leaf the photoperiodic signal of the environment (the light-dark-change) is perceived by photoreceptors. The day-length (night length) is determined in a timing system (a circadian clock). If the length is correct (for instance short day in short day plants) the photoperiodic induction occurs in the leaf. A signal is produced and (red bent arrow) transported to the apex. It switches the apex from vegetative growth to flower formation (‘evocation´). Homeotic genes, ‘floral identity genes’ and flowering time genes play a role. The apex is differentiated to a flower or a flower stand and the flower(s) develop. After [95]*

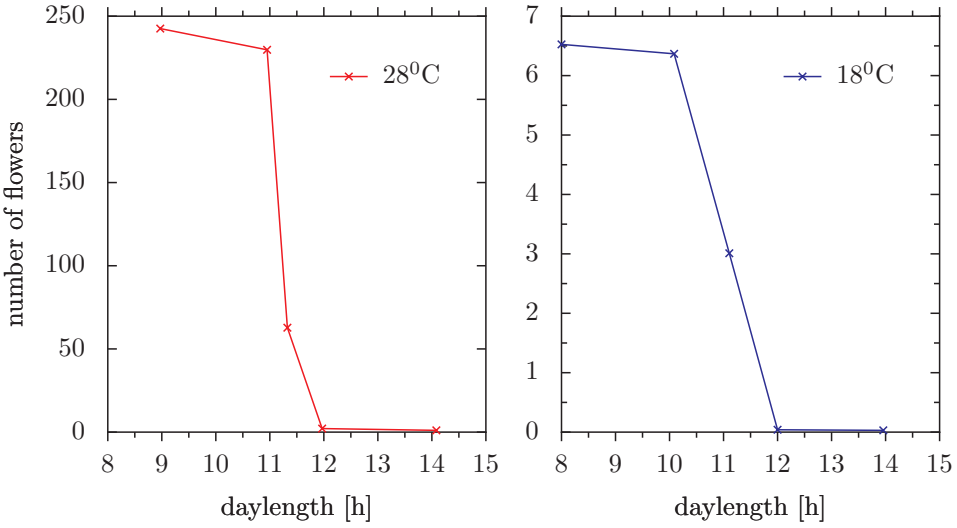

Figure 13.14: *Photoperiodic induction of flowering in* Kalanchoe blossfeldiana *at 28°C (left) and 18°C (right). Although the critical daylength has not changed (around 11 hours), the number of flowers formed is quite different (above 200 at 28°C, below 7 at 18°C). After [184]*

Temperature independence of photoperiodic flower induction

In the same way as the period length of circadian rhythms is almost independent of the temperature (*'temperature compensation'*, see page 38), the photoperiodic induction of flowering is also not much affected by the prevailing temperature. Figure 13.14 gives an example. Here *Kalanchoe blossfeldiana* plants were kept at two different temperatures (28° and 18°C) and various groups treated with different daylengths. Being a short-day plant, the groups kept under daylengths shorter than 11 hours were induced to flower, whereas longer light periods prevented flowering. However, the number of induced flowers varied considerably (40 times more under the higher temperature). Temperature compensation of the critical daylength is found also in other photoperiodic reactions besides flower induction.

Photoreceptors of the day-length

Which photoreceptors perceive the photoperiodic signals of the environment? In peas it is phytochrome (see subsection 20.13.1) which is responsible for it ([1548]). In *Arabidopsis thaliana*, however, a blue light receptor, cryptochrome, is involved besides phytochrome in order to perceive the day-length ([708], [1206], [537]). For details see page 253.

Florigen

If the critical day-length is exceeded (short day plants) or subceeded (long day plant), a flowering hormone florigen is supposed to be formed according to [217]. According to other hypotheses there are several hormones, which act together ([96]) or an inhibitor is removed. The events are complex and just now one starts to bring more light into it by using different mutants (see page).

Is there a universal flower hormone? The following findings speak in favour of it:

- If a photoperiodically reacting plant is kept under non-inductive conditions and only one or a few leaves are photoperiodically treated, the plant will flower ([1603]).
- If a stem or leaf is photoperiodically induced and grafted to a non-induced plant under non-inductive conditions, flowering is induced.
- If a leaf is photoperiodically induced and grafted to a plant, which is photoperiodically insensitive, this plant will flower earlier ([858]).
- Grafting between photoperiodically insensitive plants lead to earlier flower induction of the recipient ([977]).
- Grafting between species and orders lead likewise to flower formation under non-inductive conditions in recipients, if the donor was photoperiodically induced.

For instance, the short day plant *Xanthium* was grafted to the long day plants *Rudbeckia*, *Erigeron* and *Centaurea* and induced successfully flower formation (figure 13.15). The same was obtained if the short day plant *Nicotiana* was grafted to the long day plant *Hyoscyamus* and if the long day plant *Sedum* was grafted to the short day plant *Kalanchoe* ([859]). However, there are also counterexamples (*Cestrum diurnum* grafted to *Cestrum nocturnum*, *Phaseolus* grafted to soybean, [411]).

The flowering hormone which was claimed to exist by [1295] and [217] was

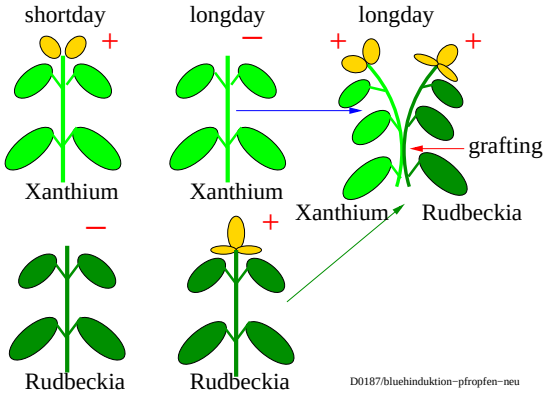

Figure 13.15: *Grafting of a photoperiodically induced short day-cutting of the short day plant* Xanthium *onto a non-induced long day-recipient* Rudbeckia *induces flowering (upper row). The* Rudbeckia *plant stays vegetative under shortday conditions (lower row). After [1107]*

intensively looked for. In spite of numerous and long studies these physiological approaches have so far not lead to convincing results. Many questions are still open such as: How many and which genes are involved in the flower induction? Which signals are used by the plant for flower induction?

More recent studies using mutants seem to be more promising (see mini review of [38]). In maize a gene was recently found, which is involved in the production or the transport of a flower signal ([239]). ID1 (INDETERMINATE1) switches mays from an indeterminated (vegetative) to a determinated condition (reproductive). In the mutant id1 the apex stays much longer vegetative (undetermined) as compared to the wild type. The mutant will finally form inflorescences. But it shows vegetative properties. ID1 is thus needed for the transformation from a vegetative to a reproductive growth. It is only in the leaves found and not in the active meristem of the stem (where the flowers are formed). SD1 is thus a part of the signal, which is transferred

from the leaf to the apex. Only mature leaves express ID1. Chimera for ID1 flower earlier as the id1-mutant, although the active meristem of these chimera was mutated. ID1 acts thus not cell-autonomously, but is involved in the transport of the signal from the leaf, which switches the meristem from vegetative to reproductive.

It would be interesting to find out whether in id1-mutants other molecular differences to the wild type are present. The flower controlling signals could well consist of promoting **and** inhibiting components.

Figure 13.16: *Development of the apex of* Pharbitis nil *under non-inductive photoperiod (long day, left) and after photoperiodic induction by short day (right). The differences are shown by macroscopic (top) and microscopic (center, bottom) pictures of the apex. After [672]*

Transport of the florigen

The flower -inducing substances are transported to the apex. It is not known whether they are the same in short day plants and long day plants. We might be dealing with organ-forming substances (inducer, [1296]) or substances, which switch on flower genes ([1603]). In mays ID1-mRNA or -protein seems to be transported as the flower signal in the conducting system of the plants from cell to cell. Or ID1 activates other genes, which express signals of the leaves (see page 255).

Switching the apex

In switching the meristem of the apex, floral meristem identity-genes ([1105]) and time-genes for flowering play a role ([1188]). The metabolism in the meristem of the apex changes. In some hours after translocation the differentiation pattern in the apex has changed. It is now stabile determined for flower induction (‘evocation´) (apex-differences after photoperiodic induction see figure 13.16).

Flower formation

Afterward the apex will differentiate to a flower and the flower will develop. Figure 13.16 shows the differences under photoperiodically inductive short day and non-inductive long day in macroscopic (top) and microscopic (center, bottom) figures of the vegetative (left) and reproductive (right) apex of *Pharbitis nil*.

Examples for short day plants: *Pharbitis nil*

In the following some examples for short day plants are presented. They are often used in photoperiodic studies, because of certain advantages. More in the examples.

The morning glory *Pharbitis nil* (figure 13.17) can be induced to flower by a single

Figure 13.17: *Morning glory* Pharbitis nil. *Left vegetative plant in long day, right flowering plant in short day*

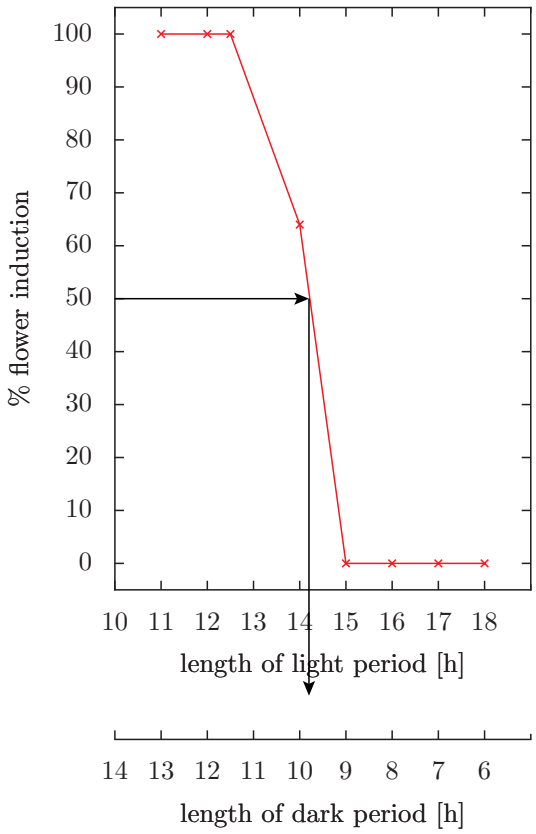

Figure 13.18: *Effect of light- and dark periods of different lengths on the induction of flower formation of the morning glory* Pharbitis nil. *After [1453]*

short day already (figure 13.18). Furthermore quite young plants in the seedling stage can be used. The changes in the apex can be seen under the binocular a few days after induction already. In this way experiments can be performed in relatively short periods of time. For special cases older plants are also useful. For instance, secondary leaves show a circadian movement which are absent in cotyledons. In this way photoperiodic flower induction and circadian events can be studied parallel to each other ([125]). Details of rearing are found in [387]. Flower reaction is measured in *Pharbitis* by determining the percentage of flowering plants, the percentage of plants with terminal flowers or the mean number of flower buds per plant.

The anatomical changes in the apex in the long day and after induction of flowering by short day are depicted in figure 13.19.

The strains of *Pharbitis nil* are differ-

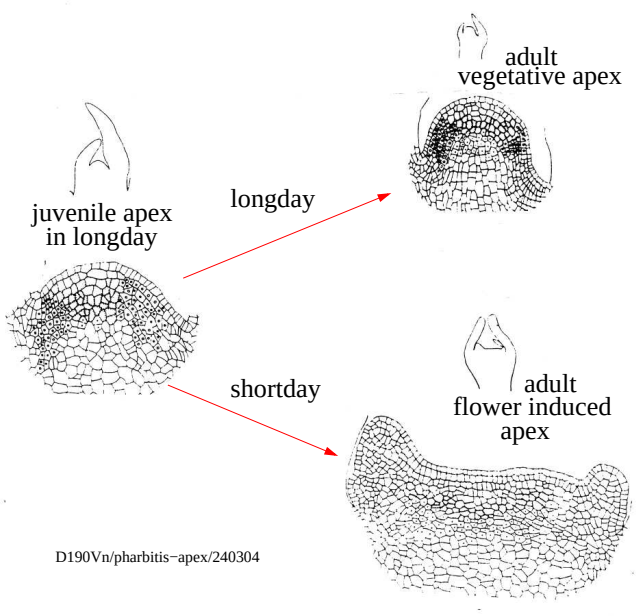

Figure 13.19: *Anatomical changes of the young apex (left) after long day (adult vegetative apex: right, top) and after short day (reproductive apex: right, bottom). Lower and higher magnification shown. After [672]*

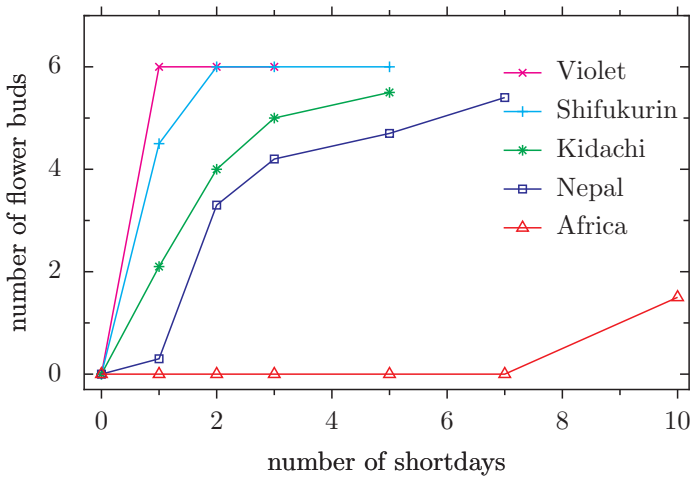

Figure 13.20: Flower reaction to different numbers of 8:16-hour short days (x-axis) of five varieties (Violet, Shifukurin, Kidachi, Nepal, Africa) of *Pharbitis nil*. After [673]

ently sensitive, depending on the geographic latitude, in which they occur, (figure 13.20). This depends on the amount of flower hormone synthesized in the leaves and how sensitive the apex reacts to the flower hormone. In *Pharbitis nil* strain violett a single short day consisting of a 16 hour dark period induces flower formation. Shorter dark periods induce also. The critical dark period for seedlings is 9-10 hours, for older plants 8-9 hours.

The physiology of flower induction in *Pharbitis* is described in reviews of Takimoto in [411] and [674]. Lumsden has studied this plant in the last years intensively ([931]). During the induction of flowering by short day the dark period plays the decisive role. But the length of the light period before the dark period, the intensity of the light and its wavelength influence flower induction also. Furthermore the age of the plants is of importance: With increasing age the plants come more easily to flower.

As in other photoperiodically reacting plants the induction *per se* is still not understood: Which signal is photoperiodically induced in the leaf and is transported from the leaf to the apex?

As a general model for induction it is proposed, that the photoreceptors receive light. After changes in the photoreceptor a photoperiodic signal affects a timing system (see figure 13.21). A photoperiodic response rhythm (PPRR) plays an important role, which has inputs from the timing system. It switches development to flower induction or keeps the plant in the vegetative stage. An external coincidence model (see subsection 13.3.15 and figure 20.25) is favored in the case of flower induction in *Pharbitis*.

Phytochrome or several phytochrome species serve as photoreceptors (see subsection 20.13.1). Light determines the phase of the oscillators and it has a direct effect on the photoperiodic process. The two effects of light are demonstrated by different dose-effect-curves (figure 13.22).

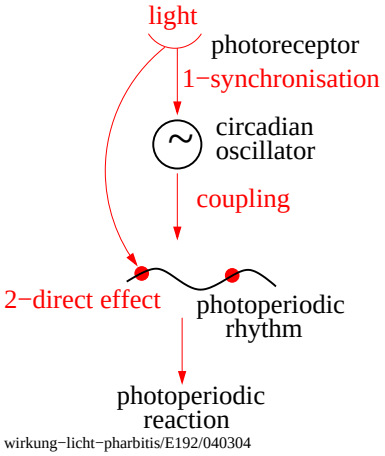

Figure 13.22: *Light has a dual effect: It synchronizes (1) the oscillator (~) which is coupled to the photoperiodic rhythm and it has a direct effect on the photoperiodic rhythm (2) by interfering with a light sensitive phase (red dots). The two effects of light are demonstrable by different doses-effect-curves. After [931]*

To understand the molecular basis of flower induction by the dark period in the leaves, a number of studies were performed ([775]). Likewise the events after induction, when the inductive signal is transported to the apex were studied ([1604]). Furthermore the influence and the effects of hormones in flower induction of *Pharbitis nil* was the aim of experiments ([1101]).

Photoperiodic experiments with *Pharbitis nil* are described in [380].

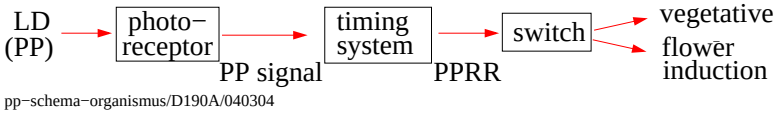

Figure 13.21: *Model of the photoperiodic induction of flowers: Photoreceptors receive light. After changes in the photoreceptor a photoperiodic signal is formed and affects a timing system. Depending on the result of this measuring procedure further physiological processes occur in form of a photoperiodic response rhythm (PPRR), which affects a switch leading either to flower induction or vegetative growth*

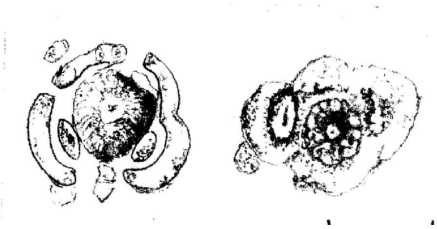

Figure 13.23: Chenopodium rubrum *looked upon from above on the vegetative apex (left, kept in long day, with leaf primordia and axillary bud primordia) and the reproductive state (right, kept in short day, ovary primordia, anther primordia, perianth primordia, leaf initials). Scala 0.1 mm. After [271]*

Examples for short day plants: *Chenopodium rubrum*

Chenopodium rubrum is a ruderal plant with early flowering. It grows at humid and even salty places. Being a 'belly plant' (you see it only when laying on your belly, [271]) it can be reared in Petri dishes for experiments, photoperiodically induced and evaluated quickly (figure 13.23). A strain from the Yukon river in Alaska can be induced to flower in the seedling stage already by a single short day (figure 13.24, [271]). Whether induction was successful can be seen already one day after induction.

Chenopodium rubrum can easily be used for photoperiodic experiments in courses and in schools ([380]).

It was assumed that the flower impulse is of electrical nature. This could, however, not be verified in more recent studies ([2]). The flowering signal seems to be more of a chemical nature.

Examples for long day plants

Hyoscyamus niger, Arabidopsis thaliana, Avena sativa (spring varieties), *Lemna gibba* G3, *Nicotiana silvestris, Rudbeckia hirta, Sedum spectabile, Sinapis alba* and *Trifolium pratense* belong among many more to the long day plants. Quantitative long day plants are for instance *Brassica campestris* and *rapa* c.v Ceves, *Hordeum vulgare, Lolium temulentum, Secale cereale* (spring variety), *Oenothera rosea* and *Trifolium pratense* cv Americ. Medium.

The number of inductive cycles for flower induction varies: A single long day induces *Anagallis arvensis, Sinapis alba, Brassica campestris* cv Ceves, *Lemna gibba* and *Lolium temulentum* to flower. Two to three days are needed by *Hyoscyamus niger*, 4 days by *Arabidopsis thaliana* and 6 days by *Silene armeria*.

The photoperiodic flower induction of the long day plant *Arabidopsis thaliana* will be treated in more detail.

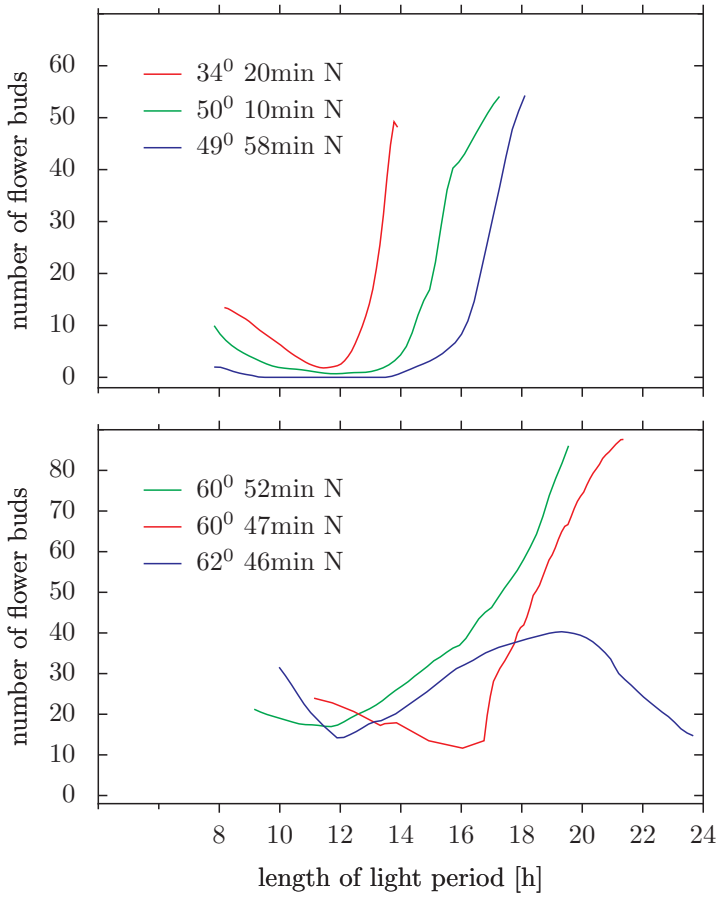

Figure 13.24: *How photoperiodic flower induction depends on day-length in six different* Chenopodium rubrum *strains of various geographical latitudes. After [271]*

Arabidopsis thaliana

Arabidopsis thaliana was introduced as an experimental plant in 1905 by Laibach (see [841]) as a suitable study object ([1061]). This plant has a number of advantages, especially a short generation time of 3 weeks only. *Arabidopsis thaliana* is small and easy to rear, has a small genome and is self pollinating.

Arabidopsis thaliana belongs to the facultative long day plants: long days enhance flowering, short days delay it, but do not prevent flowering (figure 13.25). There are various strains, which differ in their photoperiodic reactions to long day. Early, intermediate and late summer-annuals and winter-annuals exist. The earliest (Wa) flowers already 11 days after germination, the latest 112 days afterward. For induction of flowering 4 days of continuous light are sufficient. During the photoperiodic induction of flowering in *Arabidopsis thaliana* not only the duration of light plays a role, but also the wavelength and the intensity. Blue light offered continuously induces flowering. Continuous red light is without effect in *Arabidopsis*. The critical day-length is 4 hours for Wa and 5 hours for Gr. To measure the photoperiodic effect, the visibility of the first flower primordia, the time of opening of the first flowers, the number of rosette- and stem leaves can be used.

Figure 13.25: Arabidopsis thaliana *as a facultative long day plant. Top: flowering, bottom: vegetative*

Whereas physiological studies in this plant did not contribute much so far to understand the events during the induction of flowering, mutants and recent molecular biological studies were of much help. For reviews see [267], [817], [1386], [1187]. Quite a number of mutants are known, in which the photoperiodic reaction of *Arabidopsis thaliana* is changed. There are over 80 genes which affect the time of flower-

ing (*flowering time genes*). Others affect the photoreceptors of the photoperiodic stimulus. For instance, several genes were identified, which are activated by blue light and which start the morphogenetic program on the way to flower induction ([733]). There are furthermore mutants in which the transduction of the photoperiodic signals after perception of the light is influenced.

The following picture emerged, which will surely change in detail: In *Arabidopsis thaliana* the switch from vegetative to reproductive growth is delayed by different factors. This delay guarantees a certain size of the plant before flowers are formed. EMF1 and EMF2 belong to these factors. The mutants emf1 and emf2 begin to flower very early. The normal inhibition of flower induction is canceled in three ways: an autonomous pathway, a long day-pathway, and a gibberellic acid-pathway (figure 13.26). In some varieties a fourth pathway, namely vernalisation, is involved. A long period of low temperature during the winter is needed as a prerequisite for flowering to occur during the spring.

There is thus a redundancy of pathways and genes, which control flower induction (cancel the repression of flowering). The autonomous pathway is used, if short day prevails for a long time. It leads, in spite of unfavorable photoperiodic conditions, to flowering, if the plant is old enough. This redundancy might also be the reason, why so far no mutant was found, which never flowers under short day. The gibberrelic acid-pathway becomes important, if plants are under short day conditions. Gibberrelic acid is required for flowering under these conditions. The long day-pathway is used, if the plants experience long day. The mutants known so far in

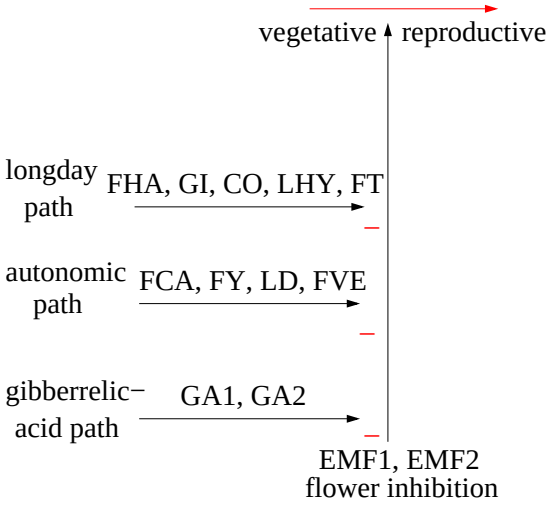

Figure 13.26: *Scheme of the different pathways, which cancel in* Arabidopsis thaliana *the inhibition of the flower induction: An autonomous pathway, a long day-pathway, and a gibberellic acid-pathway. After[267] based on proposals by [817], [818] and [964]*

which this pathway is affected, lack genes which play a role in the transmittance of the photoperiodic signal. How they are arranged between the perception of the photoperiodic stimulus and the photoperiodic reaction, namely the switch to flowering, is shown in figure 13.27.

How day-length is perceived: Several models are discussed in subsection 20.16 how photoperiod is measured by the organism. The most prominent one are the external and the internal model. Both are based on circadian clocks being involved in the measurement of day-length. However, in longday plants the duration, intensity and wavelength of light are also important for inducing flowering. A modified external coincidence model was proposed for longday plants with an internal rhythm in sensitivity to far red light

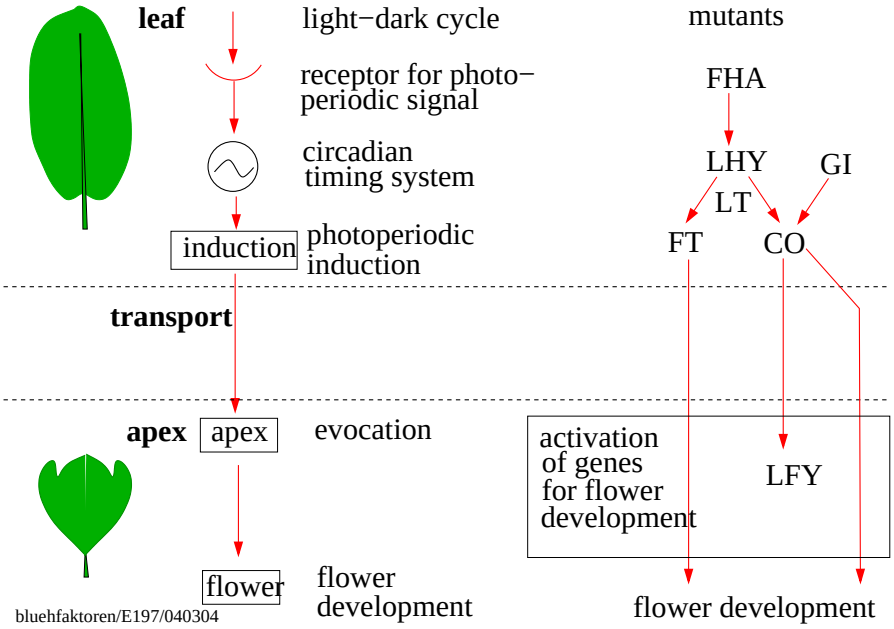

Figure 13.27: *Model of the interactions of genes, which promote flowering of* Arabidopsis *in short day. Mutations in FHA, LHY, GI, CO and FT -genes delay flowering in long day. The FHA gene codes for a blue light receptor and is active at the begin of the photoperiodically inducing step. If LHY is missing, the circadian rhythm is gone. The gene is apparently needed for the functioning of the circadian clock. In short day more CO is formed and this activates genes such as LFY, which are important for the development of flowers. GI is in the same pathway and upstream of CO. FT is also on the long day-pathway, but on a side track, which activates other genes of the flower development than LFY. After [267]*

with which the light-dark cycle interacts directly or phase shifts it. As a result the maximum in far red sensitivity coincides with the light period and flowering is promoted ([517], [1470]).

Mutants in which the perception of light is impaired, have been studied. The results show, that phytochromes and cryptochromes are involved in perceiving the photoperiodic environment in *Arabidopsis*. CRY2 seems to promote, PHYB to repress flowering (review: [897]). Thus, constant far red and blue light promote, red light inhibits flower induction. CRY2 may promote flowering by antagonizing the inhibiting effect of PHYB in red light. PHYA is also implicated in photoperiodic flowering. It may affect directly expression or activity of genes involved in flowering-time, or phase shift the circadian clock.

Besides mutants, in which the function of the photoreceptors is affected, others are known, in which the *transfer* of the light induced signals is changed. Such mutants are known both for phytochrome (see subsection 437) and for the blue light receptor.

Ultimately all the pathways up-regulate the floral identity genes needed for the development of flowers.

Most mutants, in which flowering time has changed in respect to the wild type, in which therefore the photoperiodic reaction is abnormal, possess a normal circadian rhythm. There are, however, three mutants, in which the circadian clock was affected: The mutant esd4 (shorter period, [1385]), elf3 (arrhythmic, [1601]) and lhy (arrhythmic, unpublished). As a hand of the circadian clock the leaf movement was used and a luciferase-construct (CAB:LUC), which conveys periodic luminescence to the plant. Although elf3 and lhy are both arrhythmic, the former flowers earlier, the latter later. This indicates that there is no direct connection between the circadian clock and the photoperiodic abnormality (arrhythmia) ([202]). Different explanation are available:

1. elf3 affects the circadian clock not directly, but on the input pathway light signal-clock. It is known (phyB), that changes in the transduction pathway of lights speeds up flowering. The effect would thus be independent of the circadian rhythm.

2. Since the clock of elf3 is still functioning in the light-dark-cycle, it might have a changed sensitivity to light and this could have altered the photoperiodic time measurement.

3. elf3 and lhy both stop the clock, but at different phases. In elf3 it is stopped in a phase, in which the photoperiodic reaction happens to be light-sensitive. Flower formation is advanced, since this phase prevails. In lhy, however, the clock is stopped in a phase, in which the photoperiodic system is light insensitive. Therefore flowering is delayed.

An early flowering mutant is elf3. It flowers even under short day early and does not react to day-length. This mutant is furthermore arrhythmic under continuous light in respect to the circadian leaf movement and CAB-expression. Since the rhythms are still observable under light-dark-cycles and since they occur also under continuous darkness, ELF3 is not a part of the circadian oscillator. Instead, it seems to be a component between the light receptor and the clock.

In order to understand the photoperiodic control of flowering of *Arabidopsis thaliana* better, mutants should be

available which switch off the two other pathways of flower induction, the GA-pathway and the autonomous pathway (for instance by using a double mutant ga1;fca). In this case only the long day-pathway would remain, and mutants could be looked for, which interfere with this pathway. The next step would be to determine the locations at which these newly found mutations intervene (see figure 13.27). Furthermore it must be found out, where in the plant these genes function. This could be done by genetic sectors in transgenic plants or in irradiated plants ([466]).

13.3 Diapause

Photoperiodic effects are widespread also among insects. Growth, development, form (morphs) and behavior (for instance migration) of the animals are affected. Especially well studied are photoperiodic interruptions of development. In this 'diapause' unfavorable conditions such as low temperature and dryness can be better out-lasted. There are quite a number of environmental factors such as humidity, temperature and quality of the food which signal unfavorable seasons. But photoperiod is the most reliable and most precise informant and is therefore used by many insects as a calendar of the year. Already 10 to 15 minutes difference in the day-length can decide whether diapause occurs or whether development continues. How the day-length is measured has been proposed in different models (see subsection 13.3.15).

Examples will be given how development in insects is controlled photoperiodically. Diapause might occur in all stages of an insect: In the egg, in the larval, pupal and in the adult stage. It is, however species specific, that is, genetically controlled. The photoreceptors used by the insects for sensing the photoperiod are known in some cases. How development is interrupted by diapause and which physiological and neuroendocrine mechanisms are involved has also been studied in some cases.

During diapause metabolism is low, the water content reduced, the behavior has changed and spermatogenesis and vitellogenesis is halted. In contrast to diapause *quiescence* is an immediate reaction to unfavorable conditions. It is finished as soon as the conditions become favorable again. An example for quiescence is shown by the chironomid *Polypedilum vanderplanki*. The larvae live in small exposed rock pools in parts of West- and East-Africa. During the dry season the pools dry out and the larvae dehydrate almost completely ([638]). They are, however, able to survive for many years in this condition and to sustain all kinds of brutal treatments such as short heating to slightly above 100^0C, liquid helium, one day of absolute alcohol, one week of pure glycerol.

More informations regarding diapause in [1319].

13.3.1 Pitcher plant midges

In bugs and swamps of Michigan and other States of the United States and Canada pitcher plants are found. Their leaves are can-like and partly filled with water. Emptying one of the canny leaves into a flat dish shows immediately, why they got this strange name: In the fluid hundreds of drowned insects or insect parts are found. If we cut one of the leaves open and look closely on the inner lining of the wall, we notice that they consist

of cells each with a cuticle which slightly overhangs the cuticle of the cell underneath, like tiles on the roof. The cuticula is furthermore slippery. It is an ideal mechanism to prevent insects and other small animals from escaping after they have fallen into the pitcher (figure 13.28). They finally drown, decay and serve the plant as nitrogen sources. Nitrogen is scarce in this type of biotope.

If lucky, you might find the larvae of the 'pitcher plant midge', for instance of the species *Metriocnemus knabi*. They belong to the *Chironomidae*. They live happily in the pitchers water and feed on the drowned animals. The pitcher plant midge passes through four larval stages in the water of the pitcher. After about 4 weeks (at 23^0C) the prepupae form a gelatin cocoon, a kind of pupal cradle, close to the water surface at the interior of the pitcher. Inside the cocoon the pupa is formed. After 2-3 days (23^0C) the adults eclose. They are winged, mate and the females lay eggs as packages on the water surface of the pitcher (figure 13.28). From September (35^0N) onward no prepupae are formed anymore. Instead the animals in the last (4th) larval stage enter diapause in the water inside the pitcher. The metabolism is lowered and the larvae become frost resistant. From February/March onward the diapause is terminated (figure 13.29). The animals creep out of the water and pupate at the interior of the walls of the pitcher. After metamorphosis the adults eclose, mate, lay eggs and the developmental cycle begins anew. During the summer no diapause occurs: The larvae pupate without stop in development.

In this insect diapause is photoperiodically induced and terminated. In the example shown in the figure the critical daylength lies around 13.5 hours (figure 13.30,

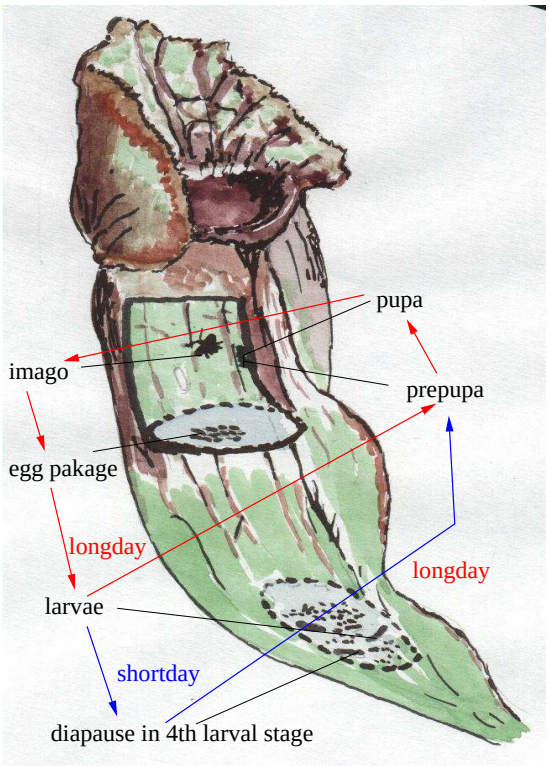

Figure 13.28: *The larvae of the pitcher plant midge* Metriocnemus knabi *live in the water inside the pitcher leaves of the pitcher plant* Saracenia purpurea. *After four larval stages in the fluid of the pitchers the prepupae form a gelatinous cocoon just above the water surface at the interior of the pitchers. In this cocoon the pupae is formed. After 2-3 days (23^0C) the adults eclose. They are winged, mate and the females lay an egg package on the surface of the water in the pitchers. After [1139]*

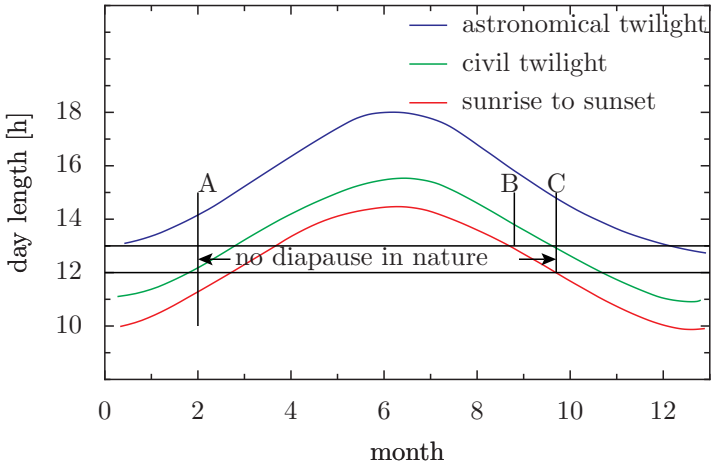

Figure 13.29: *From end of February/begin of March the first pupae are observed in nature (A with vertical line). This is the time at which diapause is terminated. Pupae are found until middle of September (B). End of September (C) no pupae are observed anymore. Between B and C is therefore the time, at which diapause occurs. After [1139]*

[1139]). It is temperature independent. 10 to 14 inducing short days are needed for a successful reaction. The sensitivity of the animals toward the photoperiodic light is extremely high: 0.00025 lux are still inducing. This might be an adaptation to the murky environment of the pitcher interior, which is additionally covered by the lid of the leaf and surrounded by *Sphagnum* mosses and other plants. Diapause in other pitcher plant midges has been studied by Bradshaw and coworkers ([145], [146], [146], [559]).

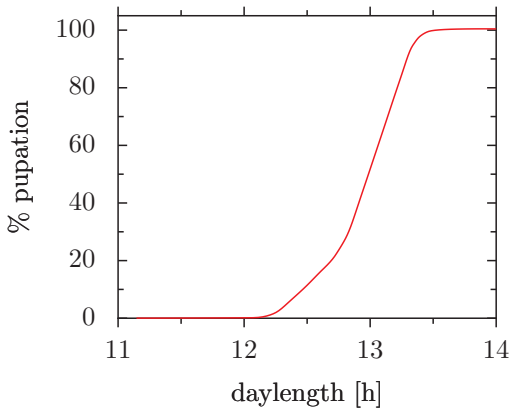

Figure 13.30: *In the laboratory it was studied, how pupae formation (that is no diapause) depends on the day-length. After 40 days at 12 hours light period all animals remained still in diapause, at 13 hours all animals had pupated (diapause terminated). The critical day-length lies thus between 12 and 13 hours. After [1139]*

Many more examples for diapause could be given. To prevent the book from becoming too thick and the reader from continuing to read it we will select just a few more examples under different views. For special literature see [1319], [72], [868], [916], [1455]; [1509] and [832] on spider mites.

13.3.2 Photoperiodism and polymorphism in aphids (*Hemiptera*)

Aphids belong to the order of *Hemiptera*, the super-family of *Aphidoidea* and the family of *Aphididae* (aphids in a more restricted sense). They are found mainly at temperate latitudes. The animals are small, unattractive and occur often in huge numbers of individuals (up to 50 000 animals per hectare). Cultivated plants and trees can be damaged by their swarming. There are several generations per year with a complicated developmental cycle. These hemimetabolic animals molt four times. The females are viviparous (give birth to larvae which emerged from the egg shell already in the mother) and in some generations parthenogenetic (but diploid). Several morphs occur, winged (*Alatae*) and un-winged (*Apterae*): They show polymorphism.

Let us follow a complete generation cycle (figure 13.31). During the fall with short days *'sexuales'* occur with males and oviparous females, which lay eggs. The eggs over-winter. During the spring the larvae eclose and develop into a *'fundatrix'*. During the longdays of the summer several generations of apterous *'virginoparae'* are produced. They multiply parthenogenetically. If the days become shorter in the fall, apterous *'oviparae'* develop (figure 13.31). Winged morphs occur under unfavorable conditions (e.g. over-population, wilting leaves. The winged animals can cover distances up to 1300 km.

[956] discovered the photoperiodic control of polymorphism in the strawberry aphid *Aphis forbesi*. Day-length is recognized via photoreceptors, which are in the brain and probably identical with neurosecretory cells (figure 13.32, [870]). These cells are also effectors and control the photoperiodic reaction. The most effective light for the photoperiodic reaction of *Megoura viciae* is blue (wave lengths from 440 to 510 nm) (figure 13.33). The precision of the day-length measurement can be quite high (up to 10 minutes!).

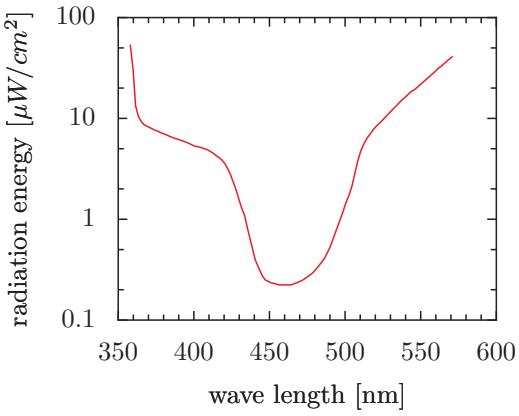

Figure 13.33: *Action spectrum of photoperiodic induction in* Megoura viciae*: The most effective light is blue (wave lengths from 440 to 510 nm). After [873]*

13.3.3 Colorado beetle

Beetles form the largest order of insects and of all animals generally. There are at least half a million species. Only in 10% of them the larval stages and the way of life are known. They are found all over the earth and on all continents. Even in the water, on glaciers, in caves and desserts they make a living.

The Colorado beetle *Leptinotarsa decemlineata* has turned to the potato not earlier than about 120 years ago. The adult is easily recognizable by its 10 black stripes on its back (figure 13.34). The females lay eggs, which develop into new generations.

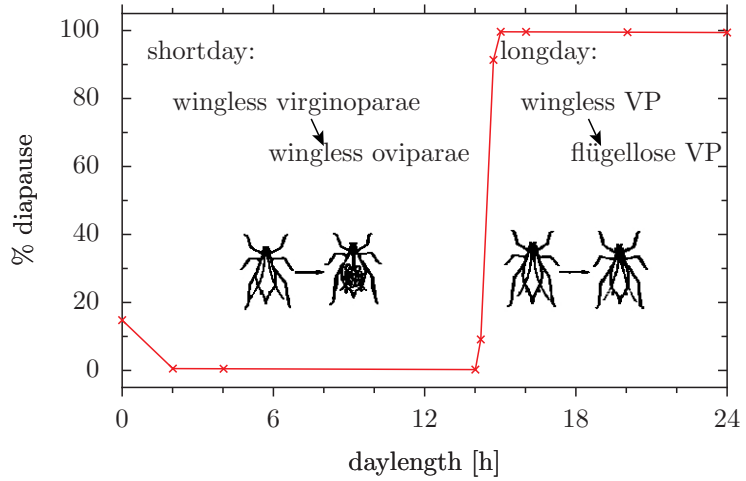

Figure 13.31: *Photoperiodic control of the generation cycle in the aphid* Megoura viciae. *In the spring larvae eclose from the eggs of oviparae females which have over-wintered. They develop into* 'fundatrix'. *These wing-less* 'virginoparae' *produce under longday conditions several parthenogenetic generations of further* 'virginoparae´ *(right part of the figure). Under short day conditions wing-less* 'oviparae' *are produced (left part of figure). The critical day-length for these two alternative reactions lies at 14 hours and 55 minutes. After [871]*

Figure 13.34: *Colorado beetle* Leptinotarsa decemlineata *on a potato plant*

When the leaves wilt in the late summer and early fall (shortday) the adults become negatively phototactic, crawl into the soil and undergo a diapause.

In a number of studies the biochemical and humoral changes before and during the diapause was followed. Quite a number of changes occur, such as a decrease in respiration, an increase in fat- and glycogen reserves, they stop feeding and the gonads become recrudescent. The animals over-winter and come again to the surface in the spring in order to find new food plants. Feeding, growth, propagation and diapause is synchronized with the development of the food plant.

On an endocrinological level it has been shown that shortday inhibits via the brain the production and secretion of the juvenile hormone of the *Corpora allata* (figure 13.35). Diapause can be induced in non-diapausing animals by removing the *Corpora allata*. Re-implantation of the *Corpora allata* breaks diapause.

Because of the damage in potato crops the behavior of the animals was also intensively studied.

Quite a number of other insects being a pest to agricultural plants undergo a diapause. To name a few, the pink bollworm *Pectinophora gossypiell*a, a pest of cotton, the corn borer *Ostrinia nubilalis*,

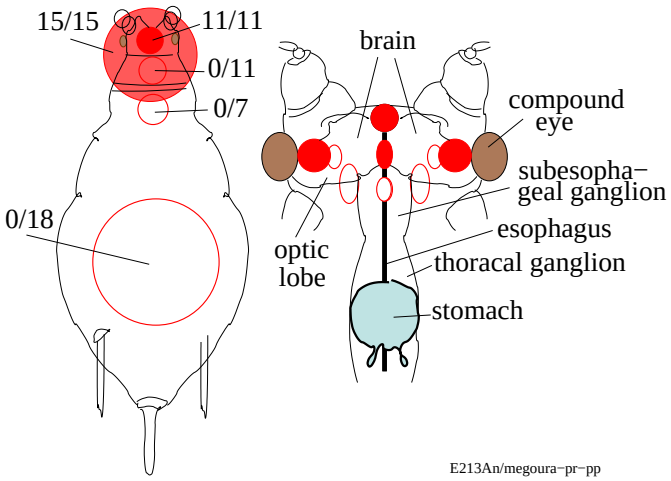

Figure 13.32: *Photoreceptors for photoperiodic control of 'virginoparae' production under long day or 'oviparae' under short day condition in* Megoura viciae. *Individual animals were treated with a basic short day of 13.5 hours. This short day was extended to a long day by adding colored light of 1.5 hours at the begin of the dark period, provided the wavelength of the light was photoperiodically effective. The additional light was directed to different parts of the body using an injection needle. The places of illumination are indicated by red circles/ellipses. In case of successful photoperiodic illumination they are filled with red. Left: Illumination of the anterior part: 15 out of 15 animals (15/15) reacted photoperiodically. Illumination of the center of the abdomen: None of the 18 animals reacted photoperiodically). The photoperiodic reaction occurred only by illuminating the head. Right: Detailed illumination of parts of the head region show, that the brain and the optic lobes are sensitive. After [870]*

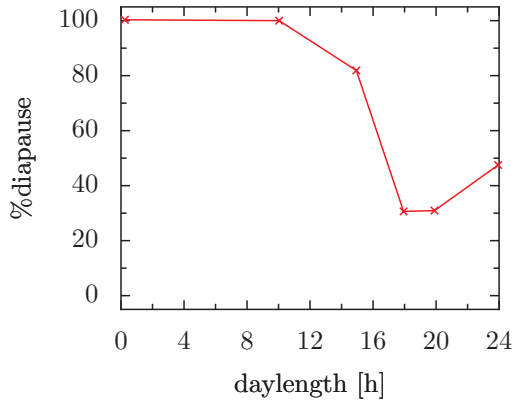

Figure 13.35: *Shortday induces diapause in the adult stage of the Colorado beetle. The critical day-length is at a light period of about 16 hours. After [319]*

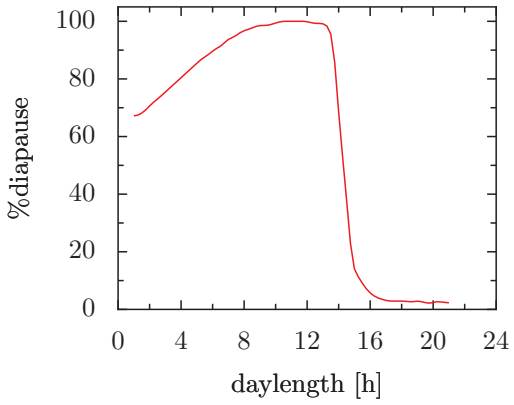

Figure 13.36: *The flesh fly* Sarcophaga argyrostoma *go through three larval stages, pupate and undergo metamorphosis. Under short day conditions development is interrupted in the adult stage before pigmentation of the cuticle. The flies are still inside the puparium and enter diapause. This stage is recognizable by the bright un-pigmented cuticle of the animals. Kept under long day conditions (critical day-length 14.5 to 15 hours) no diapause occurs, which is recognizable by the pigmented fly in the puparium. After [311] and [312]*

which originates from Europe and arrived in 1912 in the United States, the pine lappet moth *Dendrolimus pini*, which is a feared pest of pine trees.

13.3.4 Flesh fly *Sarcophaga*

The flesh fly *Sarcophaga argyrostoma* is another example for a diapausing insect. No eggs are laid. Instead, the first larval stage develops in the mothers uterus. After emerging they go through three larval stages, pupate and undergo metamorphosis (figure 13.36). In shortdays (critical day-length 14.5 to 15 hours) development is interrupted before the cuticle of the adults in the puparium has pigmented. The animals enter diapause. The photoperiodically sensitive period of development affects the (intra-uterine) embryos (after vitellogenesis) and three larval stages, especially the second and third. A certain number of short days is needed for diapause to occur (photoperiodic counter). The light receptors are in the brain. In shortdays hormone deficiency develops and as a consequence diapause is induced ([311], [312]). In longdays metamorphosis is not interrupted by diapause. The adults eclose, the females are fertilized and a new generation begins.

Whether the animals are in diapause, can easily be checked: If the puparium is opened, the diapausing animals are white, the non diapausing pigmented ([1316]). An experiment is described in [380].

Sarcophaga is a good example for the model of external coincidence ([1318]) (see page 278).

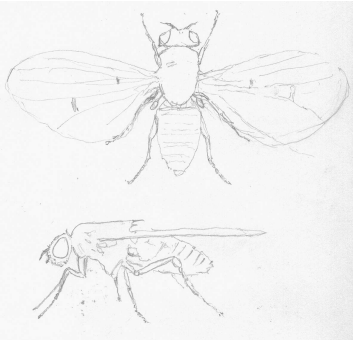

Figure 13.37: *Females of the fruitfly* Drosophila littoralis. *Drawn by the author*

13.3.5 Fruit fly *Drosophila*

Several cases of diapause were described also in *Drosophila*-species. Diapause might occur, depending on the species, in the adult stage (*Drosophila robusta, Drosophila obscura, Drosophilaphalerata, Drosophila littoralis, Drosophila transversa* and *Drosophila subobscur*a), in the pupal stage (*Drosophila alpina*) or in the larval stage (*Drosophila deflexa*). *Drosophila deflexa* remains during the winter in the larval stage and pupates not before the spring ([62]). A diapause in the adult stage during the winter was described for *Drosophila nitens* ([188]), *Drosophila robusta* ([204]), *Drosophila subobscura* ([62]). In Japan numerous species were described by Toda ([1480], [88]) and furthermore several *aurelia* -species were studied in respect to their photoperiodic reaction ([1165]). It is likely, that diapause in *Drosophila*-species in temperate and especially in higher latitudes is more wide spread as known so far.

As an example for diapause *Drosophila littoralis* is presented (figure 13.37). The females are photoperiodically sensitive. In shortday no eggs are produced. The gonads stay small. We are therefore dealing with a diapause in the adult stage. In long days the diapause is terminated (5 to 10 days needed). This species is found in Europe in different geographic varieties from northern Finland to Italy. Depending on the latitude the critical day-length differs (figure 13.38). It is 20 hours in animals from Oulu (variety 1036, 65.0^0N), 18.8 hours in animals from Inari (68.8^0N), 18 hours in animals from Paltamo (65.0^0N), 14.7 hours in a variety from Zürich (65.0^0N), and 12.3 hours in animals from Batumi (variety 1052, 41.6^0N). Animals from the Tessin (variety 1008, 46.2^0N) do not display a diapause ([861]).

Ovarian diapause is induced in females of *Drosophila melanogaster* by short days. As in most cases, the night-length rather than day-length is measured and a circadian system used. It was shown by [1322] that even in the absence of the *per* locus DNA in an arrhythmic mutant diapause is induced (see figure 13.39). That shows, firstly, that apparently photoperiodic induction and locomotor activity involve a separate circadian mechanism at both the molecular and neuronal levels. Secondly, the *per* gene is not causally involved in night-length measurement by the photoperiodic clock. However, the *per* gene does affect the photoperiodic timing, since flies in which the *per* locus is defective (per^{01}) or missing (per^{-}), an altered critical dark period is found. In these arrhythmic mutants the critical light period was about 3 hours shorter in the (per^{01}) and about 5 hours shorter in per^{-}. There was, however, no difference in the critical day-length between the wild type on the one hand and the short-period (per^{s}) and the long-period (per^{L2}) mutants on the other hand.

For both processes, photic entrainment of the circadian clock and photoperiodic

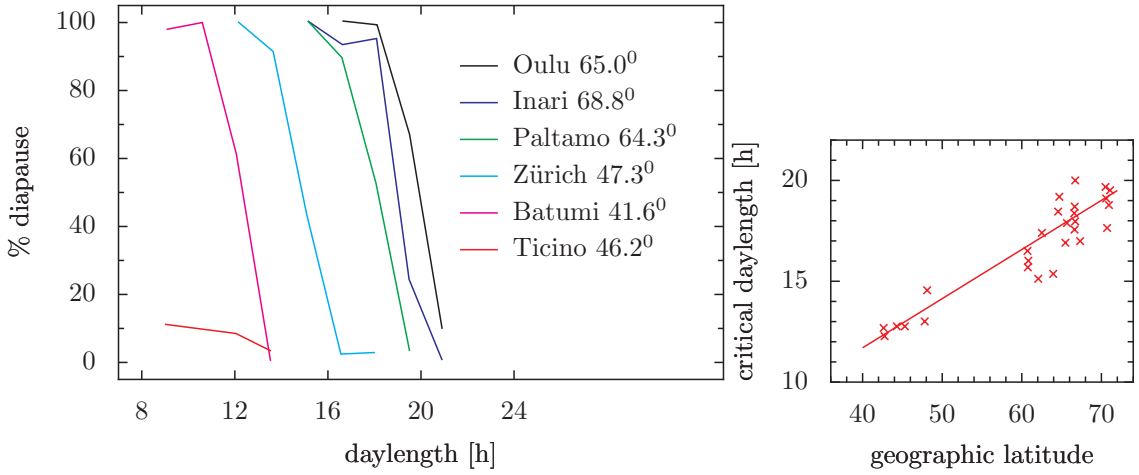

Figure 13.38: *The critical day-length increases in the different geographical varieties (left curves) of* Drosophila littoralis *with higher latitudes (40 to 70°N, right part). For locations see also right part of figure 13.55). After [861]*

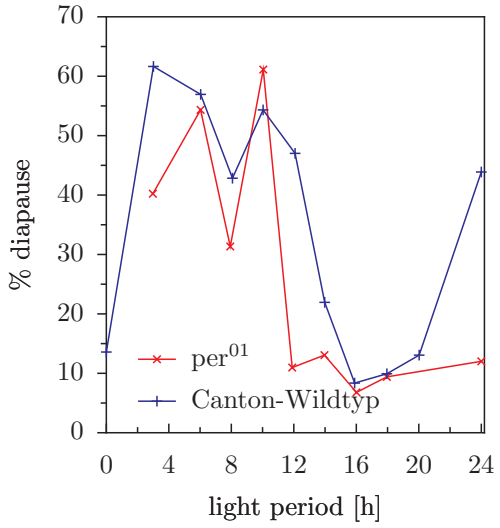

Figure 13.39: *The* per *gene affects photoperiodic timing: Flies with defective* per *locus (per⁰¹) possess a critical light period about 3 hours shorter as compared to the wildtype. After [1322]*

time measurement, the compound eyes and ocelli are neither essential nor necessary. The responsible extraretinal photoreceptors and the circadian clock are not within the optic lobes, but in the central brain. Lateral neurosecretory cells serve as the pacemakers and as photoreceptors. The cellular substrate of the photoperiodic mechanism may involve the pars intercerebralis region of the mid-brain.

The genetics of the diapause of various varieties of *Drosophila littoralis* was studied by a group in Oulu (Finland) ([861]). The factor for diapause is segregated as a single Mendelian unit (x-chromosome, in the neighborhood of the white-locus). It is variable enough, to explain the different critical day-lengths (12 to 18 hours) in the different geographical varieties. Diapause is dominant over non-diapause ([*930*]). In the measurement of day-length neuronal and humoral events are involved, as shown in *Drosophila grisea* ([736]).

13.3.6 Diapause in the silk moth

As a final example the diapause of the Chinese silk moth *Bombyx mori* is presented. It belongs to the insect order of *Lepidoptera* and there to the family of giant silk moths (*Bombycidae*). It is found in the tropical and sub-tropical regions of Asia mainly.

The life cycle of the animals and the photoperiodic control are illustrated in figure 13.40. *Bombyx mori* possess a diapause in the egg stage. Silk moths are, in contrast to many other diapausing insects, longday-animals. The females lay eggs in short days during spring. They develop without diapause. In longdays, however, females develop, which produce diapausing eggs ([982]). Diapause begins in a certain embryonic stage and continues until the temperature has dropped for at least 14 days to 5^0C. The low temperature terminates diapause. During diapause no cell division is taking place (it is halted in the G-2 phase) and the development of the embryo is interrupted.

Differences between shortday-animals and longday-animals were looked for and found: The longday[4] induces in females of *Bombyx mori* a signal in the neurosecretory cells of the brain, which reaches by neuronal pathways the subesophageal ganglion. There a diapause-hormone is produced and secreted (figure 13.41).

Via the hemolymph it reaches the ovary and prevents the embryos from developing. Instead they enter diapause ([1051]). The diapause-hormone is a neuropeptide consisting of 24 amino acids. It was cloned ([1586], characterized ([1589]) and synthesized ([668], [1300]).

The diapause-hormone-gene is expressed in the subesophageal ganglion of pupae and pharate adults, but not in other tissue. It was localized to 12 neurosecretory cells in the neighborhood of the ventral mid line of the subesophageal ganglion. They are organized in three groups ([1312]), produce the diapause-hormone and project to the *Corpus cardiacum* ([666]). The natural and the synthetic diapause-hormone induce the expression of the trehalase gene in the developing ovaries. The trehalase mRNA increases 4 hours after the injection by a factor of 6 ([668], [1432]).

The reactions during the chilling period (5^0C, over-wintering) and afterward are well known biochemically due to work of Kai and coworker ([732]). The duration of the chilling period is measured by an esterase, EA4 (Time-interval-measuring-esterase, an ATPase). After 14 days of chilling the EA4 is activated, both in vivo and in vitro, the cells enter rapidly the S-phase and as a result the development of the embryo continues ([731]). EA4 presents thus a kind of molecular timer (figure 13.42).

Which mechanism activates this time measurement? A certain component, PIN (‘peptidyl-inhibitory needle´) is needed. It is a peptide with known amino acid-sequence ([679]) and presents a factor which holds time ([729]). It forms an equimolar complex with EA4 which prevents EA4 from becoming active. At low temperatures PIN dissociates from EA4. At 5^0C this takes 14 days[5] and consists of a series of conformational changes of EA4 by using a built-in mechanism in the

[4] The head capsule of *Bombyx mori* contains in the pupal stage an opaque triangle. It allows light to reach easily the photoreceptors in the brain ([138])

[5] if the eggs are transferred to low temperature after oviposition. If chilled 10 days after oviposition, it takes 50 days

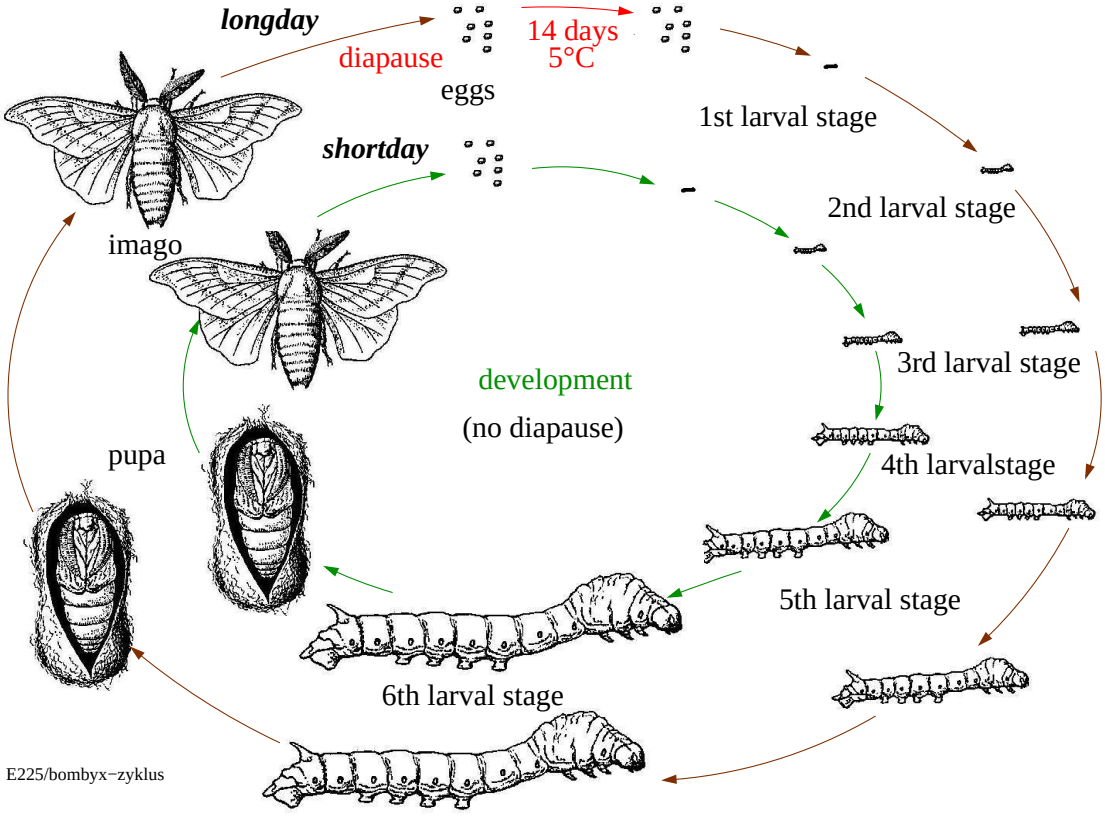

Figure 13.40: *Females of the silk moth lay eggs under short days, which develop without entering diapause (inner circle, green arrows). Larvae emerge from the eggs, which molt six times. After the last molt the animals pupate in a cocoon which they spin. Under long day conditions females develop which produce diapause-eggs (outer circle, brown arrows). They over-winter. Diapause occurs in a certain embryonic stage. In order to continue development the embryos have to be chilled for twelve to fourteen days at a temperature of* 5^0 *C or lower (red arrow). This starts a process ('alarm clock'), which terminates diapause at higher temperatures and reactivates development (green arrows). After [678]*

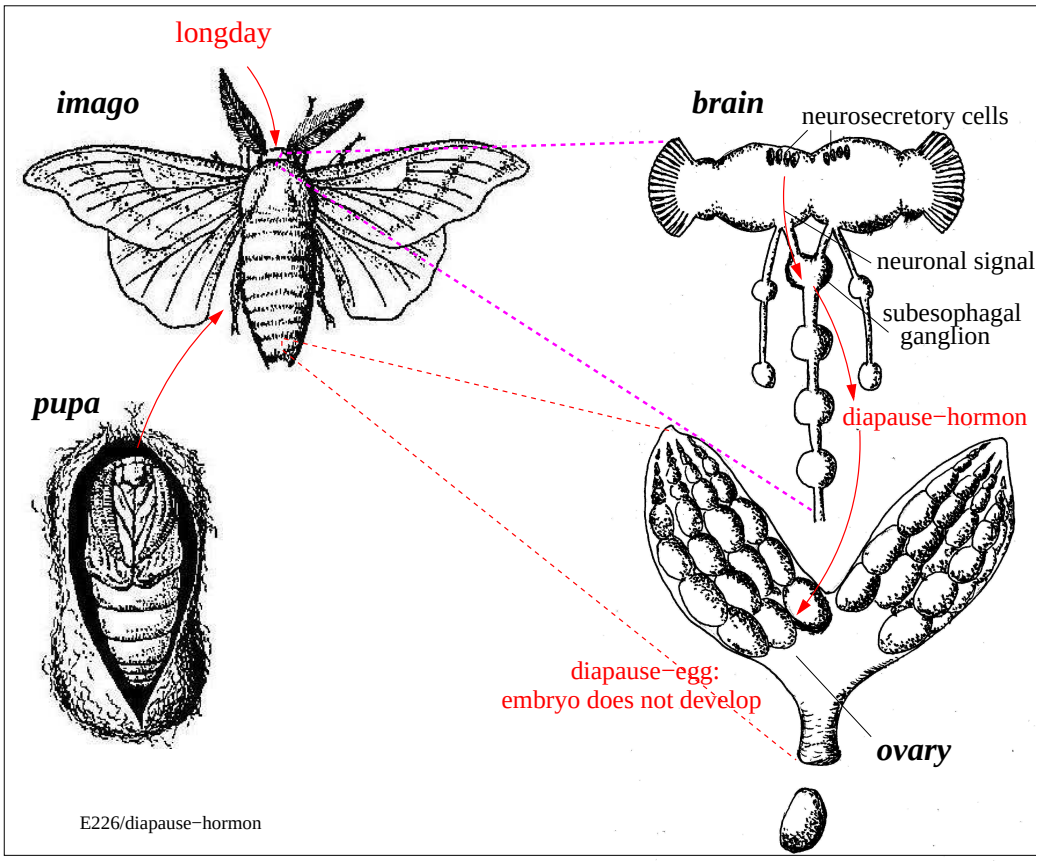

Figure 13.41: *Emerged females of* Bombyx mori *or females still refined to the cocoon are induced to lay diapause eggs under longday via neurosecretory cells in the brain. A signal is transferred by neurons from the neurosecretory cells to the subesophageal ganglion. In the lateral part of the subesophageal ganglion (arrangement see right part of figure, after [1312]) are diapause-hormone-producing cells. From here the diapause-hormone (a neuropeptide consisting of 24 aminoacids, [1587], [1312]) reaches via the hemolymph the ovary. It stimulates trehalase (an enzyme responsible for glycogen synthesis), inhibits esterase A4 (which activates the yolk proteins for the development of the embryos) and increases the permeability for 3-hydroxikynurenin (which leads to the formation of ommochromes and pigmentation of the diapause-eggs). Finally the diapause-hormone stops the development of the embryos in the diapause-egg ([678])*

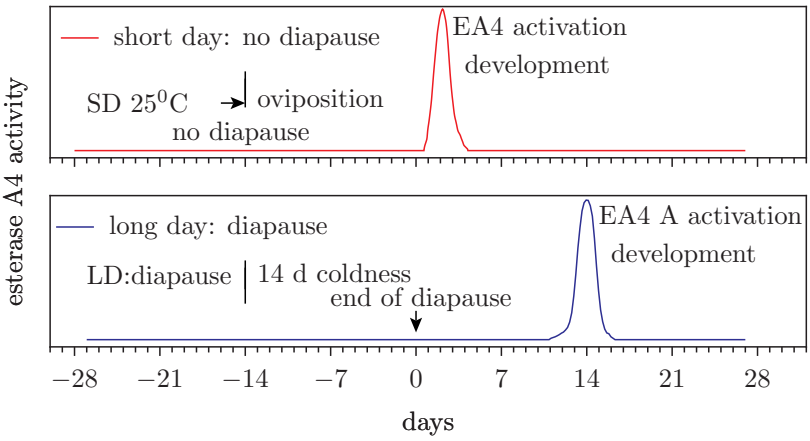

Figure 13.42: *Under shortdays the females of* Bombyx mori *lay eggs, the embryos of which possess a high activity of the esterase A4. They develop at* 25^0C *via larval stages into an adult. Under long days the esterase A4 is inactive. A chilling period of* 5^0C *or lower is needed (black bar below the x-axis), to activate the esterase A4. It terminates diapause and the embryos develop at higher temperatures. At* 25^0C *larvae eclose 14 days after the end of the low temperature-period. The esterase A4 (EA4=TIME, Time-Interval-Measuring-Esterase) measures the duration of the chilling period by becoming intramolecularly modified and activated in this way. This occurs in vivo and in vitro. EA4 is thus a molecular timer and alarm clock. After [732]*

protein structure. If transferred to 25^0C EA4 becomes suddenly active for a short time([730]). Afterward the EA4 activity decreases again. As a result, the embryo cells enter rapidly the S-phase.

Instead of using low temperature PIN can be removed with a Sephadex gel filter and EA4 activity is detectable after 7 hours in 25^0C already ([729]). In the presence of PIN EA4 is heat stable. Heat does not disturb the time measurement ([732]).

This example demonstrates a long-term hourglass-type timer. It accurately times a developmental switch at the end of diapause after a fixed duration. It is not known yet how EA4 leads to a resumption of embryogenesis. EA4 is a glycoprotein which contains a N-linked oligosaccharide. The carbohydrate seems to be essential for the regulation of the EA4 time measurement through the interaction with PIN ([1454]). Other developmental timers are known (for instance [618]).

13.3.7 Characteristics of diapause

After these examples for diapause a few general remarks are appropriate. Diapause is a strategy of many insects and mites to survive unfavorable seasons ([288]). It is a state of dormancy where development is stopped or drastically reduced, metabolism is low, reproduction arrested, behavior altered and the animals become resistant to cold, heat or drought. In contrast to quiescence diapause begins before the environmental conditions become unfavorable. And it ends not before *diapause development* has come to an end even if the environmental conditions have become favorable again.

Diapause is in most cases induced by the photoperiod (temperature, food, moisture might also play a role in certain cases). Photoreceptors (see subsection 13.3.12) receive the photoperiodic signal and discriminate light from dark. The length of the night (the length of the day is seldom used) is determined by a system which might use a circadian clock for this purpose. A photoperiodic counter adds up the number of photoperiodically effective cycles. After a minimum number the informations are send to a center which processes the integrated information and controls the photoperiodic events at the target organs (figure 13.43). In only a few cases diapause is also finished photoperiodically. Usually other conditions such as a certain period of low temperature or internal processes are needed for terminating diapause.

13.3.8 Different types of diapause

In many areas of the temperate and higher geographic latitudes the winter is unfavorable for the development of organisms generally and of insects in particular. Therefore a winter diapause occurs in these cases. In other areas of the earth dryness is the limiting factor. This is especially true for desserts. Here summer diapause prevails. The photoperiodic conditions leading to winter diapause are shortday, and those leading to summer diapause are longdays ([1317]).

In insects, the post-embryonic development of which lasts a year or several years (univoltine species), diapause occurs in each animal in a predefined developmental stage. This type of diapause is called obligatory. In multivoltine species with several generations per year the diapause is facultative: It occurs only in the generation in which the external conditions induce diapause (for instance shortday in the fall) (figure 13.44). In some insects

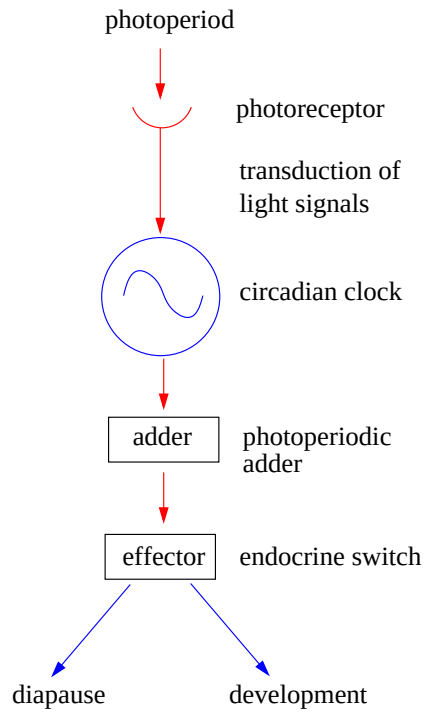

Figure 13.43: *A photoreceptor perceives photoperiodically effective light. Signals reach a timing system, a circadian clock. A photoperiodic counter adds up these signals. Having reached a threshold, photoperiodic induction occurs in a center: An endocrine switch controls the photoperiodic events at the target organs. This leads, depending on the photoperiod and reaction type, to development or to diapause (stop of development) of the animals*

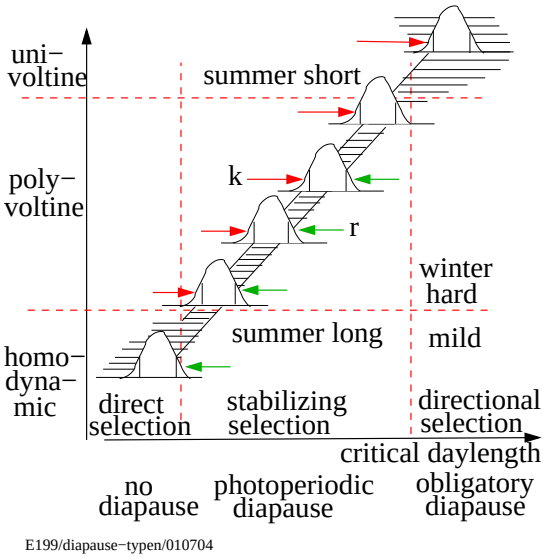

Figure 13.44: *Obligate diapause occurs in univoltine species in a certain developmental stage. In multivoltine species with several generations per year the diapause is facultative: It is found only in the generation in which external conditions induce diapause (for instance short day in the fall). After [928]*

such as *Bombyx mori* varieties exist which possess an obligatory diapause and others which possess a facultative diapause ([678]).

13.3.9 Diapause stages

Diapause can occur in the pupal, adult, egg or one of the larval stages. In which stage the animal diapauses is characteristic for the different species. In the examples of diapause we have already seen some realizations (page 262 to page 268).

Larval diapause occurs mostly in the last larval stage. But exceptions are known: In *Choristoneura funiferana* it is the second larval stage, in which diapause is found ([573]), in *Dendrolimus pini* diapause can be observed in different larval stages

([484]).

The photoperiodically sensitive stage is mostly before the stage in which diapause occurs. For example, young larval stages can be photoperiodically induced, whereas the diapause occurs only in the last larval stage. In the silk moth *Bombyx mori* the adult female is photoperiodically sensitive. The reaction to the photoperiodic signal occurs after the larva has become a female which produces a diapause hormone. This hormone stops the embryo in the egg from developing (see subsection 13.3.6). In the giant silk moth *Philosamia cynthia* the larvae in the 4th and 5th stage are sensitive to shortday. In *Diataraxia oleracea* the last larval stage is photoperiodically sensitive for two days only. More in [1317].

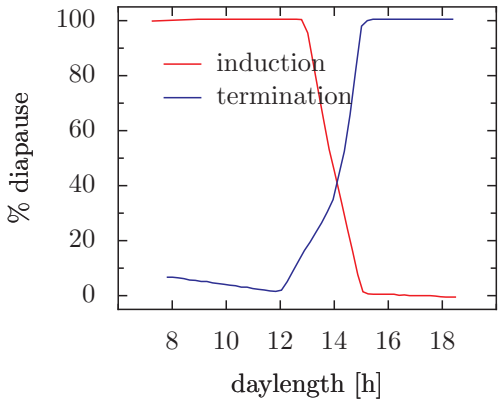

Figure 13.46: *Short days during fall induce diapause in the oak silkworm,* Antheraea pernyi, *whereas the increasing day-length of the spring terminates it. The critical day-lengths of both events are identical. The photoperiodically sensitive stages are the last larval stages and the pupal stage. After [1317], [1567]*

13.3.10 Geographical varieties

Since the onset of unfavorable conditions in the different areas is variable, we find also differences in the critical day-length in varieties of different geographical latitudes. Such ecotypes exist for instance in *Acronycta rumicis* (upper part of figure 13.45). Here the differences in critical photoperiod are gradual. In *Pieris brassicae* however only two geographical varieties are found (lower part of figure 13.45). The genetics of such varieties is especially well known in *Drosophila*-species ([861]).

13.3.11 Induction and termination of diapause

In a number of insects diapause is induced and terminated photoperiodically in the same stage. The giant silkmoth *Antheraea pernyi*, the corn borer *Ostrinia nubilalis*, the pitcher plant midge *Metriocnemus knabi* belong to it. The short days of the fall induce in the corn borer diapause in the pupal stage. The increasing days in the spring terminate the diapause (figure 13.46). Photoperiodically sensitive are the last larval stages and the pupal stage. In other insects diapause is induced already in stages long before the actual resting stage. In this case diapause is frequently broken by other factors than day-length, for instance by a longer period of low temperature.

In the oak silkworm *Antheraea pernyi* the diapause is also photoperiodically induced and terminated. The critical day-lengths are identical for both events (figure 13.46). This indicates that the same timing system participates in both reactions. The wave lengths 400 to 500 (blue and green) are photoperiodically the most effective ones. The photoreceptor is located in the brain. On top of the brain a translucent window is found in the cuticle of the pupa. Light can penetrate through it eas-

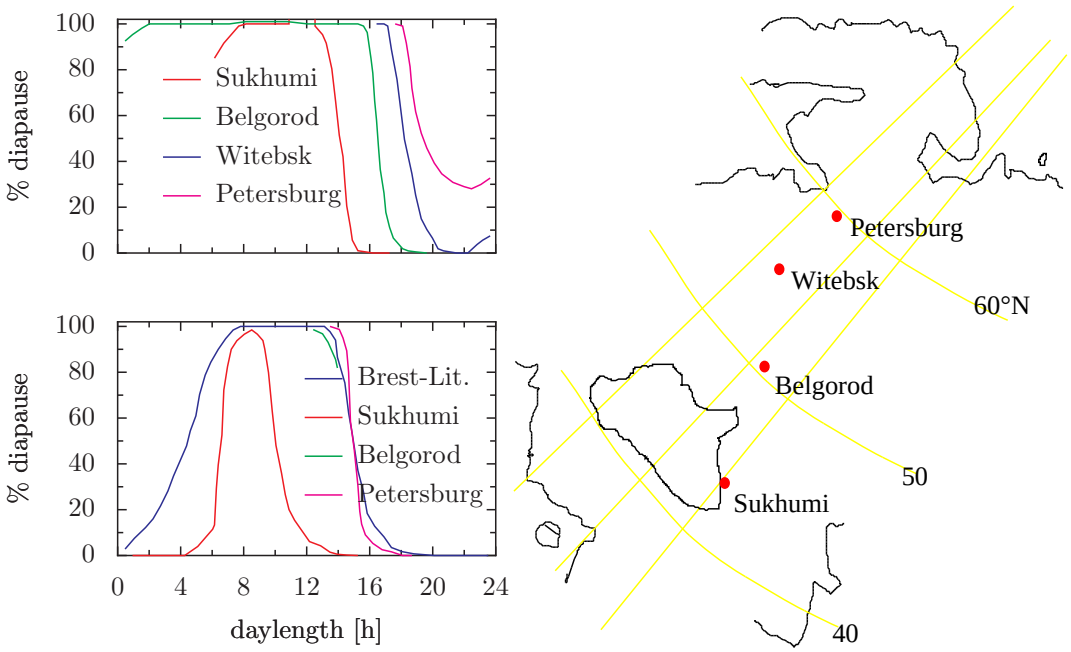

Figure 13.45: *Geographical races of* Acronycta rumicis *(top) and* Pieris brassicae *(bottom) from different geographical latitudes (right). Percent diapause as a function of day-length. Right: Locations of the races studied. After [287]*

ily. This plays, however, normally not a big role. It becomes important if the cocoon happens to be in a shadowy place. More important is the cocoon which is an ideal light collecting sphere.

The critical day-length tells us at which length of the light period 50% of a population is photoperiodically induced. In the case of the diapause-induction of the giant silkmoth it is 14.2 hours (see page 13.46). In longer light periods less animals or none would enter the diapause, in shorter more or all. For the *termination* of diapause the critical day-length of the giant silkmoth is also 14.2 hours.

13.3.12 Photoreceptors

To measure the day-length light receptors are needed. Photoperiodic sensitivity begins usually during the twilight between values of 10 to 100 lux. In this range the changes in light intensity per time unit are maximal under field conditions (figure 13.47). The photoreceptors can, however, be shielded from the environmental light to different degrees. In this case the optical peculiarities of the cuticle, the cocoon, the hiding place during the winter play a role.

Candidates for such photoperiodical photoreceptors are the compound eyes, ocelli and light sensitive organs or structures of the brain. In the various cases of diapause in insects different photoreceptors are used (figure 13.48). The efficiency of different wavelengths was determined in a few cases ([1098]).

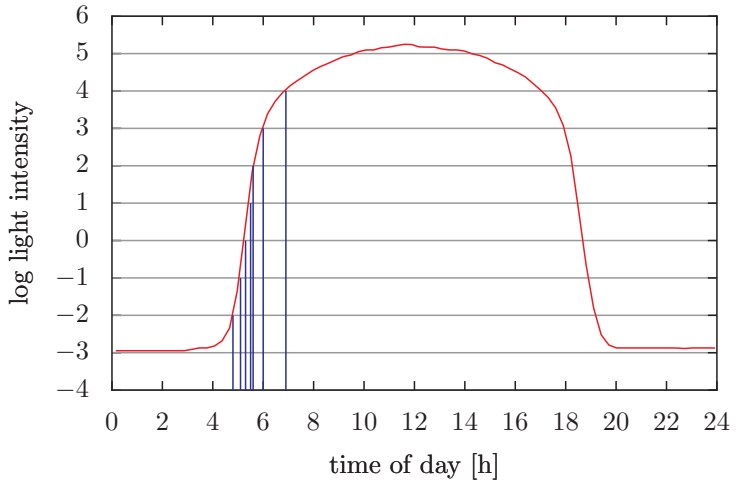

Figure 13.47: *Time course of outdoor light intensity recorded on the second April 1966 in Tübingen, Germany (48^{0}32′N, 9^{0}3.5′E). Clear weather, new moon. During twilight the intensity changes are maximal between 10 and 100 lux. After [401]*

13.3.13 Timing system

After the photoperiodic stimulus has been received, a timing system has to determine the day-length and to induce accordingly (shorter or longer than the critical day-length) a sequence of processes which finally lead to the diapause state. A number of models exist, but only few substantial ideas.

With light pulses given at various times during the dark period it was shown that there is one -in other cases two- sensitive phases for photoperiodically inducing light. In figure 13.49 from 0:00 CT (circadian time) onward the processes A, afterward B, C, D, E and F occur. If light hits the system during process C, the photoperiodic decision *development* is made. Later (for instance in E and F) factors are produced which bring about development. If no light occurs at C, the decision *diapause* is made.

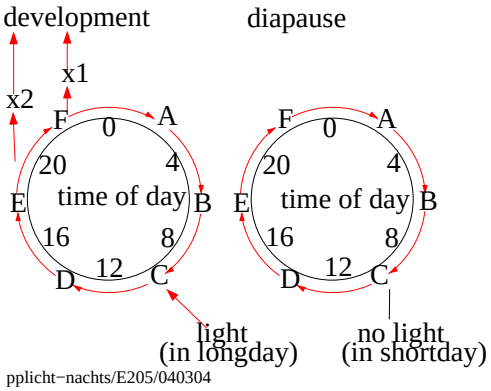

Figure 13.49: *From circadian time 0:00 onward process A, afterward B, C, D, E and F occur. If light is present during process C, the photoperiodic decision* development *is made. Afterward (for instance during E and F) factors are produced which lead to development. If light is absent during process C, the decision* diapause *is made. After [276]*

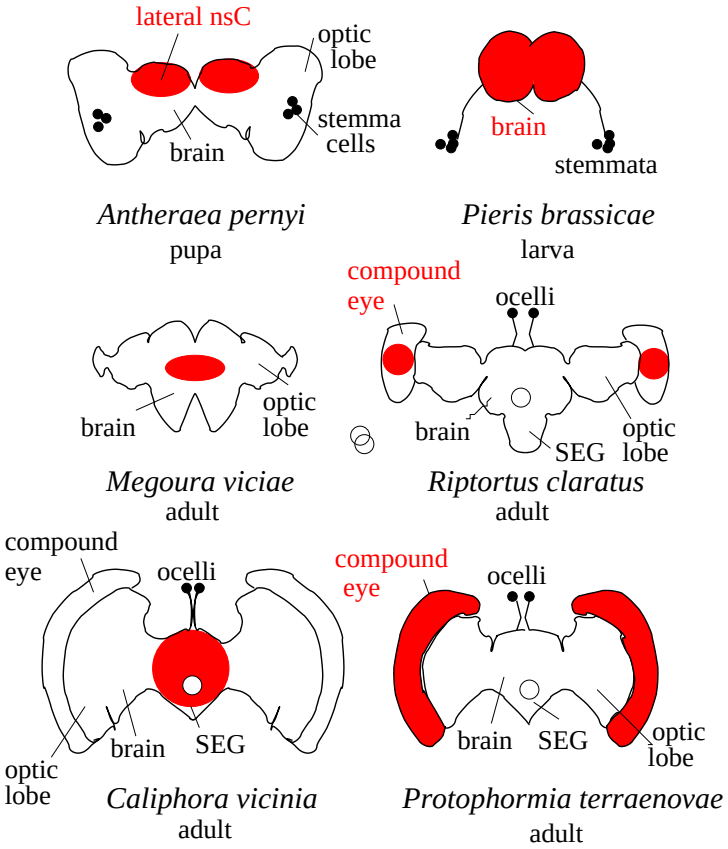

Figure 13.48: *Photoperiodic receptors were localized in the areas marked red in six arthropods (frontal view of brain, optical lobes and eyes). After [1098]*

13.3.14 Photoperiodic counter

In some cases of induction of diapause a single inductive cycle is sufficient as for instance in *Chaoborus americanus* ([143]). In most cases, however, several inductive cycles are needed.

The number of cycles needed depends only slightly on the environmental temperature (Q_{10} of 1.04), whereas duration of lifetime, larval development and oviposition rate show a normal temperature dependency with a Q_{10} of 2.7. In the cabbage moth *Mamestra brassicae* the short days, in the aphid *Megoura viciae* the long days, in *Acronycta rumicis* both are counted (see photoperiodic counter chapter in [1317]).

The parasitic wasp *Nasonia vitripennis* is especially suited for studies on the photoperiodic counter: The mother is photoperiodically sensitive and transmits the photoperiodic effects via the eggs onto the larvae. The eggs are daily deposited. Thus the physiological condition of the mother can be followed by observing the kind of offspring (diapausing or not) (figure 13.50).

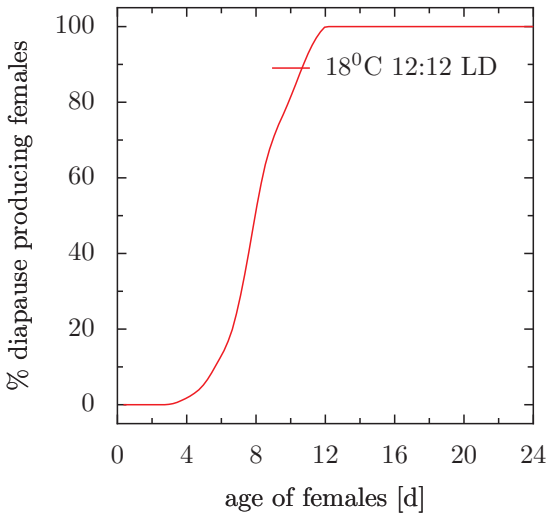

Figure 13.50: *Photoperiodic counter in the parasitic wasp* Nasonia vitripennis. *The mother is photoperiodically sensitive and transmits photoperiodic signals to the eggs and the larvae. Eggs are daily deposited which allows to monitor the physiological state of the mother. After [1314]*

13.3.15 Models

For the photoperiodic time measurement different models have been proposed (discussed in [1317], [1455] and under special topics in subsection 20.16). They rely either on an hourglass principal[6] ([872]) or on a circadian oscillator ('external coincidence', [180]) or on two oscillators which interact with each other ('internal coincidence'). A more recent model of [887] takes also care of the number of cycles,

[6]The day-length is measured similar to the way an egg clock used to function, where fine sand falls from one glass bulb through a thin bottleneck into another glass bulb.

which are needed for diapause induction. With this model the different forms of photoperiodic response curves and the results of more complicated light programs such as the Nanda-Hamner- and the Bünsow-experiments can be simulated. The double role of the light, namely to synchronize the photoperiodic timing system *and* to influence the photoperiodic reaction, is also simulated with this model. Other models such as the internal coincidence model are for instance not able to explain the low photoperiodic reaction in very short light periods and in continuous darkness. According to the model presented here these effects would be the result of a damped circadian oscillator: A factor is summed up, until a certain number of days (required day number RDN) reaches a threshold, which allows induction (figure 13.51). The counter can be either a compound which due to the photoperiodic treatment promotes the reaction actively, or a compound, which due to the photoperiodic treatment inhibits the reaction. Both was found and in both cases neurosecretory cells are involved. An endocrine effector converts these signals into the diapause reactions.

13.3.16 Physiological basis, endocrinology of the diapause

Diapause is characterized by certain adaptations and switches in the metabolism. Thus, substances like glycerol and sorbitol in the hemolymph are produced, which serve as an antifreeze. Storage substances such as fats, proteins and carbohydrates are formed. Wax prevents the cuticle from drying out. How are these adaptations and changes induced?

The photoreceptors, the time measuring system and the photoperiodic counter of the photoperiodic induction of diapause of insects are all in the brain.

In many insects species with diapause in the larval-, pupal- and nymphal stage the developmental arrest is brought about by a hormonal deficiency (example for larval-pupal diapause figure 13.52). The brain - prothoracic gland -system is inactivated. The day-length is perceived by neurosecretory cells in the brain. In the case of a diapause-inducing day-length (for instance short day) the brain does not produce the brain hormone (=PTTH, prothoracotropic hormone)[7], the prothoracic gland does not synthesize ecdyson. This leads in the giant silk moth *Hyalophora cecropia*, in *Ostrinia nubilalis*, in *Pieris rapae*, and in *Sarcophaga* to the diapause characteristics such as low metabolism, low water concentration, high fat content, a changed behavior (a cocoon is for instance produced). As a result, development is interrupted.

In other cases in which larvae enter diapause, the endocrine system continuous to be *active* (figure 13.53). The larvae can molt, but not pupate. Pupation is prevented by juvenile hormone. The brain triggers the Corpora allata to produce and secret juvenile hormone. To allow larvae to molt, the prothoracic gland must function and secret ecdyson. An example is the corn borer *Diatraea grandiosella*.

In the *larval diapause* the metabolism is reduced, the body contains little water, fat is stored, metamorphosis inhibited, the locomotor activity reduced. Partly larval molts continue to occur. Development is reactivated by temperature or photoperiod.

[7] The brain hormone is a large, nondialysable heat resistant molecule. Its molecular weight is above 10 000.

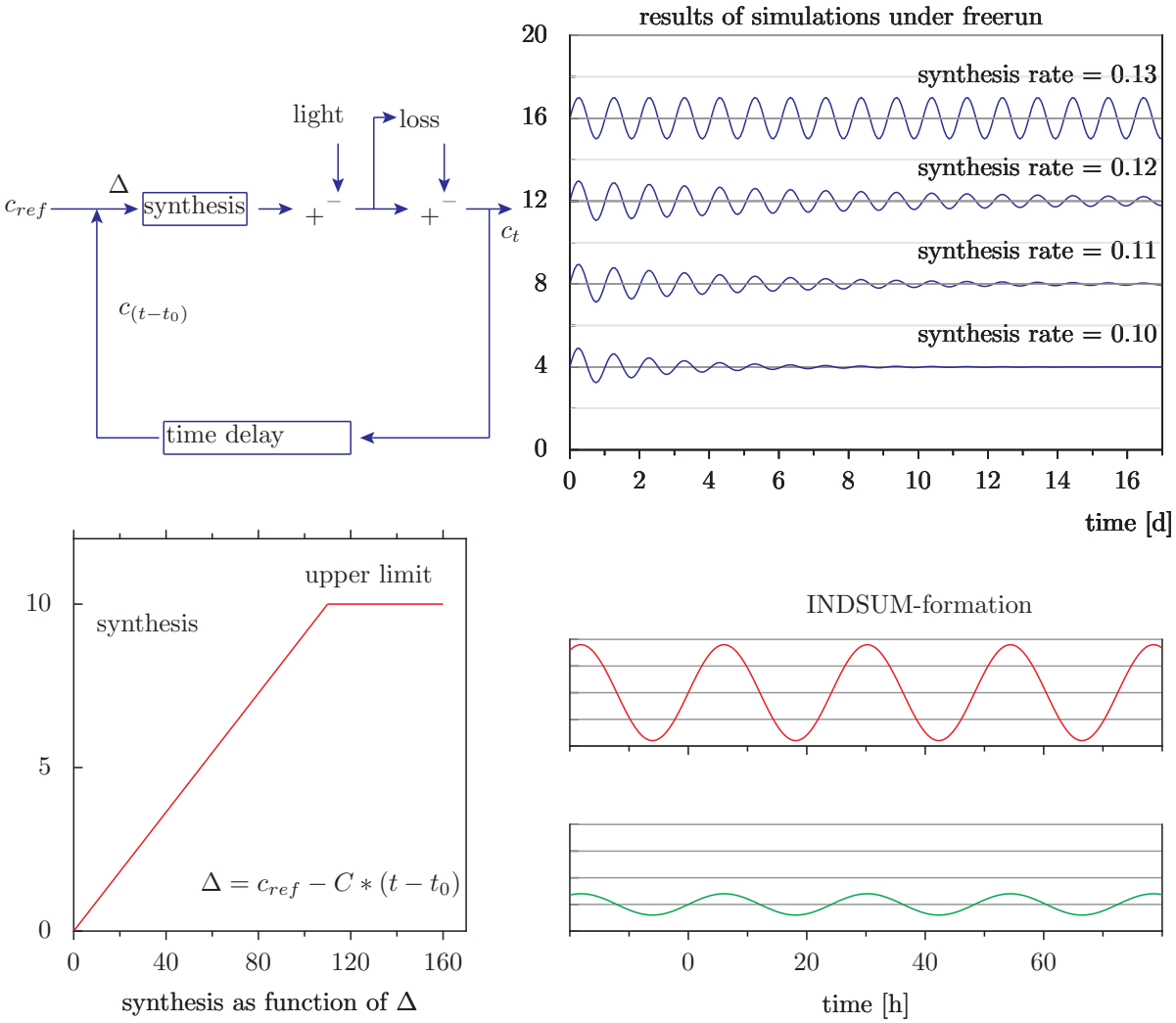

Figure 13.51: *Model of photoperiodic counter by Lewis and Saunders. Top left: Control system of feedback oscillator. Synthesis of the oscillating substance c_t is regulated by the difference between the reference value c_{ref} and the time delayed values of c_t $c(t - t_0)$. Light adds to the concentration of c_t, while there is always a loss and a time delay. Top right: Depending on the synthesis rate SR simulations lead to the blue curves. Lower rates bring about stronger damping. Horizontal lines are thresholds, and c_t values above threshold are summed up over time (INDSUM formation, bottom right). Dynamics of synthesis rate shown in bottom left diagram as a function of the $c(t - t_0)$ value. Synthesis rate is limited by an upper border, to prevent the amplitude of the oscillations from becoming too high (bottom left). After [887], [1323], [1324]*

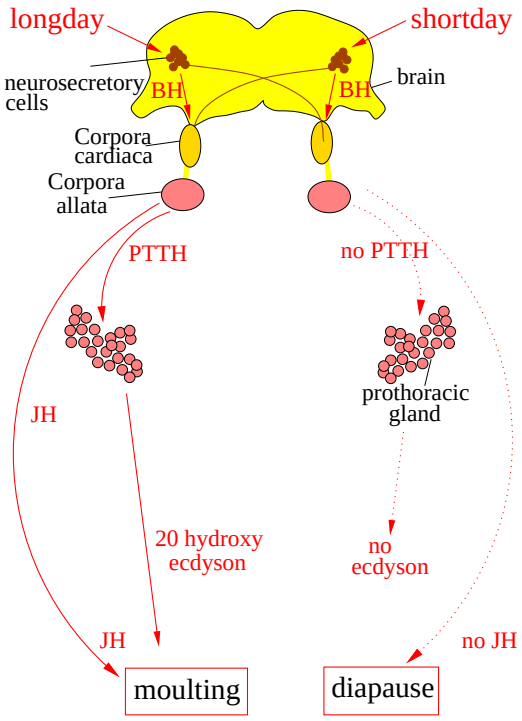

Figure 13.52: *During diapause in the larval- and pupal stage (for instance in* Hyalophora cecropia, Ostrinia nubilalis, Pieris rapae, *and* Sarcophaga*) a developmental arrest is induced by lack of hormone: The brain - prothoracic gland - system is inactivated. Day-length is perceived by neurosecretory cells in the brain. At a day-length which induces diapause (for instance short day) the brain does not produce brain hormone, and the prothoracic gland does not form ecdyson. Development is arrested. After [1566]*

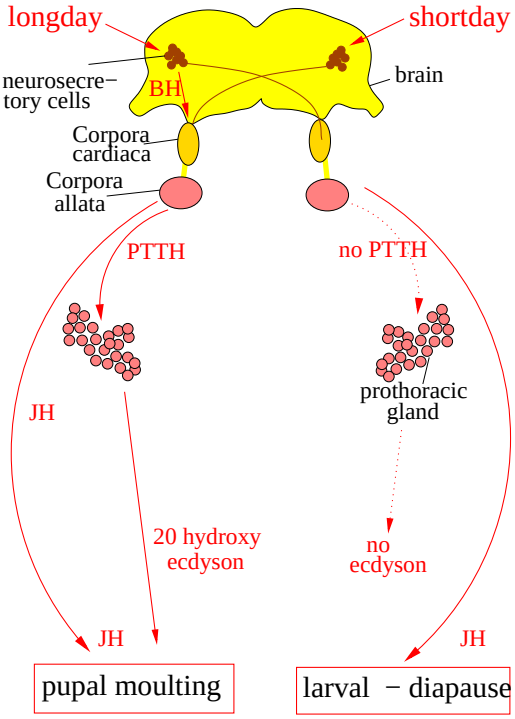

Figure 13.53: *Diapause of the corn borer* Diatraea grandiosella *during the larval stage: The brain induces the Corpora allata to produce and secret juvenile hormone. However, the prothoracic gland does not secret ecdyson. The metabolism is reduced, the body contains little water, fat is stored, the locomotor activity reduced. Metamorphosis is prevented. After [1596]*

During the *diapause in the imaginal stage* neurosecretory cells are inactivated, which control the *Corpora allata*. The inactive *Corpora allata* does not produce juvenile hormone anymore and therefore the ovaries are inhibited. This is for instance the case with the Colorado beetle (see subsection 13.3.3). Parallel to it the behavior changes. The adults become negative phototactic, stop feeding and crawl into the soil. If the *Corpora allata* are removed, diapause is induced. If the *Corpora allata* are implanted into diapausing animals, they develop in spite of shortday. Shortday thus inhibits the production and secretion of juvenile hormone, the reproduction is prevented and diapause begins. Ecdyson-production is normal.

In the *egg diapause* of the silk moth *Bombyx mori* the photoperiodic signal *longday* is received by the mother and a diapause hormone of the subesophagal ganglion inhibits the development of the embryos in the egg during blastokinesis (around the middle of the embryonic development). During shortday, however diapause of the embryos is prevented and the animals continue to develop (see subsection 13.3.6).

Most frequently diapause occurs in the *pupal stage*. This is true especially for *Lepidoptera* and *Diptera*. Here too the metabolism is reduced and the mitochondria are less active. Imaginal differentiation does not take place.

The pupae are usually not photoperiodically sensitive anymore (with other words, photoperiodic induction of the pupal diapause occurs during a larval stage). But here too exceptions exist: In *Hyalophora cecropia* and *Antheraea pernyi* the pupal stages are still photoperiodically sensitive. Diapause can be prolonged during the pupal rest, and interrupted by longday. In *Platysamia cecropia* the hormonal events are more closely known (see subsection 13.3.6).

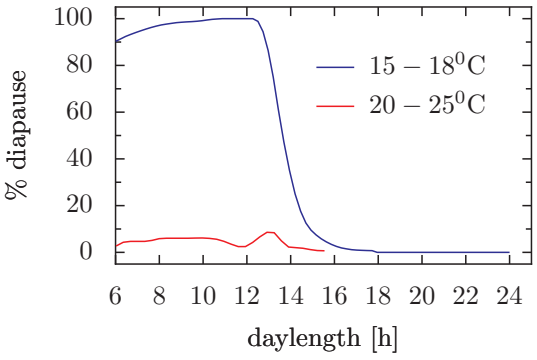

Figure 13.54: *Diapause of* Sarcophaga *at various temperatures. At* 20^0C *and higher temperatures diapause does not occur anymore. After [1315]*

13.3.17 Modification of diapause

Diapause can be modified by factors such as temperature and food availability or quality of food. Usually high temperature prevents diapause, whereas low temperature promotes it. In some cases the critical day-length decreases with increasing temperature. In *Megoura viciae* it is for 5^0 C higher temperature decreased by 15 minutes, until at 23^0C diapause does not occur anymore ([869]). In *Sarcophaga*, however, the critical day-length stays constant at varying temperatures, but does not occur at 20^0C and higher temperatures (figure 13.54).

On the other hand there are also cases in which diapause occurs at higher temperatures, for instance in *Abraxas miranda* ([965]). The animals develop in shortday and at lower temperatures. In most cases optimal temperatures exist for the photoperiodic induction of diapause. Tropical species exhibit usually a higher optimal

temperature (*Oedipoda miniata* $27 - 28^0$C).

Food can influence diapause too. At a high content of oil of the host plant (cotton seeds) the diapause of the bollworm *Pectinophora* is facilitated. In *Chaoborus* a high food supply can suppress diapause in spite of shortday ([144]).

13.3.18 Genetics of diapause

Diapause of insects is genetically programmed. It shows a certain variability, which substantiates for instance in examples of geographical varieties with different critical day-lengths (figure 13.38). Bastards between a northern variety of *Drosophila littoralis* (Oulu) and a more southern variety (Kutaisi) show intermediate behavior (figure 13.55, [929]).

13.4 Photoperiodic control of reproduction in mammals

For reviews see [647], [1416], [596] and [506].

13.4.1 Introduction and overview

Mammals in the temperate and higher latitudes have to adapt to the seasons, in order to ensure that the offsprings are born and reared at favorable environmental conditions. The temperatures of the surrounding and food supply change drastically during the course of the year. Therefore numerous other functions such as thermal insulation by the pelage have to be adjusted accordingly.

Small mammals with short gestation periods such as voles, mice, hamster and ferrets mate and deliver their litter during the spring and summer. Larger mammals such as sheep, goats and deer or mammals with delayed gestation (bats, minks and badger) mate in the fall or winter and deliver their offsprings in the following spring. The photoperiod is the most important environmental factor, which synchronizes the annual rhythm of reproduction. In mammals which mate during the spring, the gonads develop at this time of the year (*'recrudescence'*), whereas under short day the gonads regress (*'regression'*). In mammals on the other hand, which mate during the fall or winter, short day induces development of the gonads and long day regresses. Body weight, fur color and -quality and body temperature regulation are also under photoperiodic control.

Photoperiodic reactions will be presented in two examples, the Syrian hamster and the Djungarian hamster. The different adaptations to the winter conditions are presented next. As an example, torpor will be explained and its adaptive value discussed. However, these adaptations might vary and exceptions to the average behavior of the population are found. We then ask our self, how mammals perceive day-length, how these informations are passed on and how they decide, whether short day or long day prevails. This is the place where the circadian clock mechanism comes into play and where different models are discussed which try to explain the photoperiodic time measurement. Finally the photoperiodic reactions must lead to changes in the target tissues, the organs and the endocrine system. By this, the reproductive system, the body temperature-control, thermal insulation and behavior are affected.

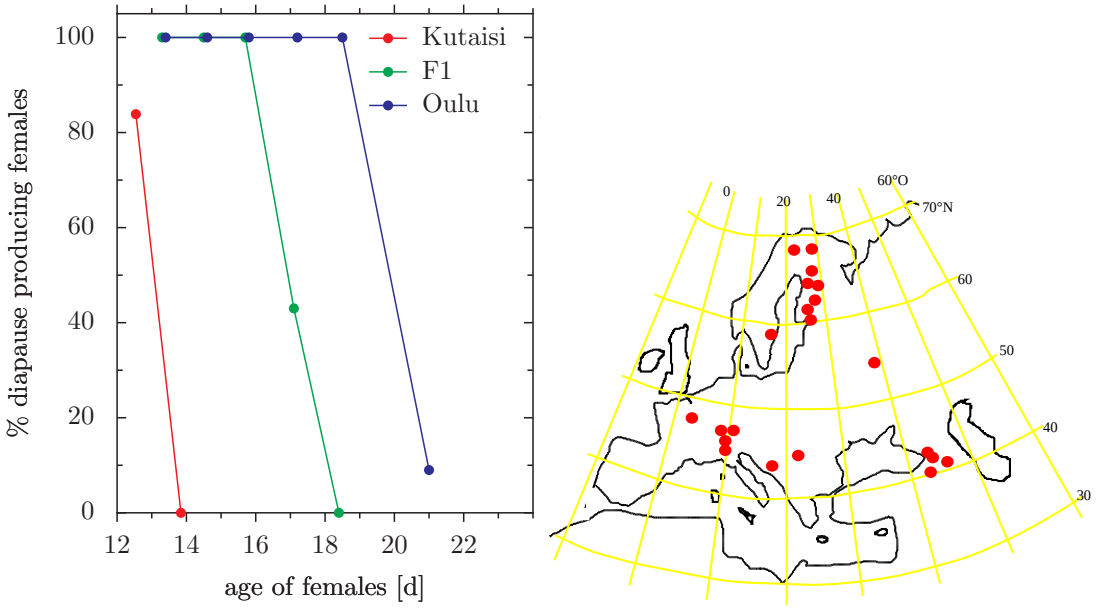

Figure 13.55: *Hybrids (F1) between a northern variety of* Drosophila littoralis *(Oulu, Finland, 65^{0}N) and a southern variety (Kutaisi, Caucasus, 42^{0}N) with intermediary critical daylength (Oulu: 19 h 42 min; Kutaisi 12 h 36 min; F1 16 h 18 min). Right: Locations. After [929]*

13.4.2 Selected examples: Syrian and Djungarian hamster

As examples for the photoperiodic control of reproduction two hamster species are presented, the Syrian and the Djungarian. Both are easy to rear and to propagate and both are well studied. Its small size allows to keep them in larger numbers in rooms with temperature and light control. Not only the photoperiodic control of reproduction, but also that of fur color, fur density, body weight, body temperature, torpor and hibernation was studied. However there is also an endogenous annual rhythm, which influences these processes (chapter 12.4). Often the photoperiod is used to synchronize the annual rhythm to the environment.

Syrian hamster, systematics, occurrence, habits, adaptation to winter conditions

The golden hamster or Syrian hamster *Mesocricetus auratus* belongs to the family of the *Cricetidae* (figure 13.56). It was originally captured in the surrounding of Aleppo. After the second world war it was introduced to Europe and became also a laboratory animal. The estrus cycle takes 4-5 d in the golden hamster. This is the time needed for the uterus to get prepared and to induce the reproductive system to nourish the fertilized egg. On the day of ovulation the females, if provided with the opportunity, run for a long time (up to 16 km, usually only 1 km). After mating it takes only 16 days until offsprings are born and they quickly mature.

During a photosensitive phase the go-

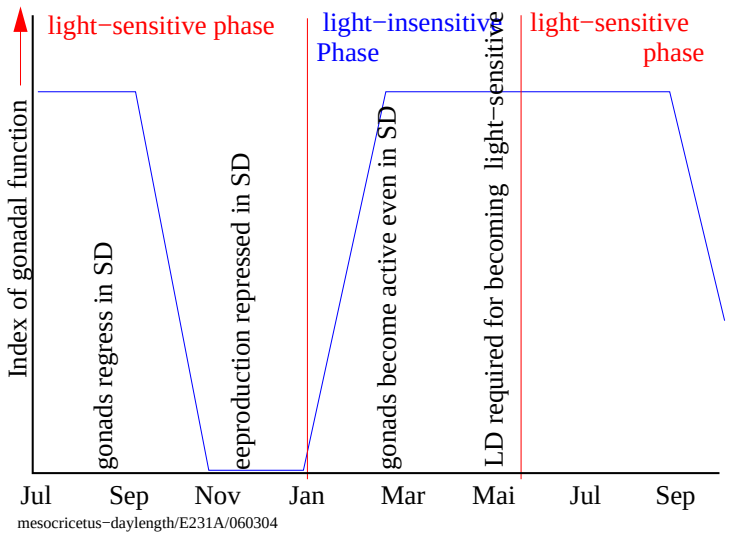

Figure 13.57: *Effects of daylength on Syrian hamster* Mesocricetus auratus. *During photosensitive phase gonads (index of gonadal function x-axis) regress in September under short-day conditions. Continued short day conditions suppress reproduction (blue curve). Whereas still in short-day photosensitive phase comes to an end (January) and photorefractory phase begins (between red vertical lines). Gonads begin to develop until fully active in March. Exposure to long-day required to re-develope photosensitivity to short-day conditions. After* [506]

Figure 13.56: *Golden hamster (=Syrian hamster)* Mesocricetus auratus (Cricetidae). *Body length 17 to 18 cm*

nads regress in the fall under short-day conditions (figure 13.57). Continued short day conditions suppress reproduction. Furthermore it retards also puberty in hamsters born late in the year, induces torpor and hibernation, changes the pelage and the body weight. Under long day conditions the gonads develop. The critical day-length is 12.5 hours. In the Djungarian hamster it is 13 hours. Whereas still in short-day (January to March) the photosensitive phase comes to an end and a photorefractory phase begins. Apparently an endogenous interval timer is set in motion by the previous short-day exposure. The gonads of the hamster begin to develop until fully active in March. Exposure to long-day is required to re-develope photosensitivity to short-day conditions ([506]).

Phodopus: Occurrence, habits

The dwarf hamster *Phodopus sungorus* and *Phodopus campbelli* are often mixed up, because both have been termed 'Siberian hamster' or 'Djungarian hamster' (figure 12.14). However, they differ from each other clearly. For instance, the effect of a short day on reproduction is less well expressed in *Phodopus campbelli*, the litter size is larger and more of the offspring survive ([352]).

Adaptations to the winter

The short days of the fall induce in the Djungarian hamster adaptations for the coming winter. Body weight is reduced, the gonads are regressed, the fur becomes white and dense[8], torpor occurs, at which

[8] It isolates better as compared to the brown summer fur and has at low wind speeds a higher heat resistance ([1526]).

the body temperature falls temporarily to lower degrees. Low temperature amplifies the short day effect and now fewer short days are needed for the photoperiodic effects to occur.[9] After some time in short day regression is terminated and the gonads develop again, body weight increases and the summer fur is formed (figure 12.14). This *'recrudescence'* starts already under short day conditions (figure 13.58). It is therefore not a photoperiodic reaction, but (probably) an endogenous annual phenomenon. It occurs in males and females ([884]).

The photoperiodic events are induced by the melatonin secretion of the pineal. A single long day is able to decrease melatonin secretion permanently. Apparently the circadian oscillator is reprogrammed by this treatment ('reset') which has thus a long term effect. Melatonin application prevents this effect ([429]). The melatonin-producing neuronal network has an effective light memory ([883]).

Torpor and its physiology

Small hamsters are able to regulate the energy balance during unfavorable conditions by a special state called torpor. Torpor helps to individually control food consumption and the search for food. The energy consumption is strongly reduced during torpor ([89], [90]). Environmental temperature and food availability are important factors in the induction of torpor. The animals are thus able to react in a flexible way to combined environmental stimuli and unpredictable weather changes ([1278], [1276]). The main factor allowing torpor to occur is however short day.

Torpor of the Djungarian hamster is also under photoperiodic control. Under short day and at low environmental temperatures the body temperature is lowered by 5.4 hours (0.3 to 9.4 hours) per day to $14 - 31^0$C as an average (figure 13.58)[10]. Energy consumption is reduced considerably during torpor. In this way the animals are able to search for food during the Siberian winter without spending more energy as if they would spend all day in their burrow [1276].

The longer the animals were kept under short day, the more frequent torpor occurred. After 130 days of short day a maximum of torpor occurrence was found. Males show torpor more frequently as compared to females. A circadian rhythm controls the timing of torpor and the day-night-cycles synchronize its rhythm. In continuous darkness the frequency of torpor increases ([779]). Torpor occurs under short day only, if the testicles have regressed. Injection of testosterone prevents torpor completely. The annual rhythm which controls the torpor, is however not influenced by the testosterone injection. In castrated animals the torpor continuous for a longer time ([1121]). Castration between the first week before and the fourth week after onset of short day treatment promotes the occurrence of torpor ([1120]).

Sleep, torpor and hibernation were understood so far as homologous events. However, in the Djungarian hamster the slow-wave activity (with an EEG power density of 0.75-4.0 Hz) is increased during torpor. The same is found after sleep deprivation. During torpor the animal seems to be under sleep deprivation. It has to compensate for it by an increased slow-

[9] Catecholamine seems to be involved in this temperature effect ([596]).

[10] During hibernation body temperature can drop to almost 0^0C ([58]).

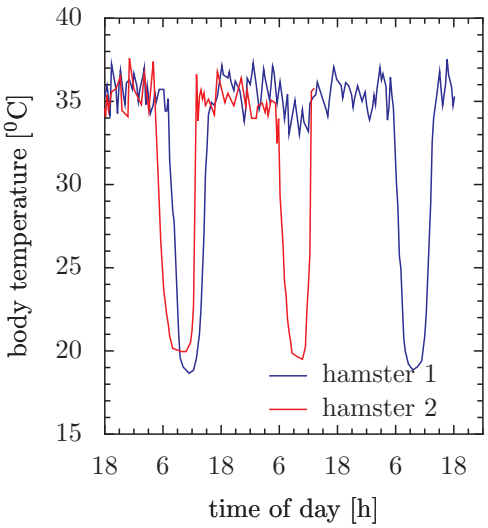

Figure 13.58: *Torpor in two Djungarian hamsters. Body temperature of hamster 1 (red curve) recorded for two, of hamster 2 (blue curve) for 3 days. Both hamsters in winter fur, hamster 1 recorded from January 18 to January 20, hamster 2 recorded from November 14th to 17th. Chilled room with* 6^0C *environmental temperature and 80 lux light from 6-18o'clock and 0.2 lux light at the remaining time. The animals become lethargic (torpor) in the early morning and drop their body temperature for some hours per day to* $18 - 20^0C$ *(hamster 2 on the second day only a few degrees). After [428]*

wave activity ([301]). There are further differences ([1120]).

Exceptions

A number of exceptions are observed during events which are under photoperiodic control in hamsters. The adaptation to the winter conditions for instance can vary in this species considerably ([1278]). Normally the Djungarian hamster reacts to short day with a number of physiological and behavioral changes as mentioned before. But not all animals react alike to the photoperiod and some animals do not react at all. It is assumed ([791]), that the circadian system is responsible for the photoperiodic reaction, but that an additional system (reduced responsiveness to melatonin, [660]) is involved, the strength of which varies genetically.

[513] showed, that the photoperiodic prehistory plays an important role for the reproductive regression. 92% of the animals kept in a long day of 18:6 hours light-dark did not regress the gonads completely, whereas only 10% of the animals kept in a 14:10 hour light-dark cycle did not regress. In none of the animals kept in a short day of 10:14 hours regression was prevented.

Photoperiodic time measurement, models

If the behavior and physiology of an animal is adapted to the seasonal changes during the course of a year, a seasonal cue is needed. The most reliable one is the length of the daily light period (or dark period) which changes in an exact way during the year. These changes are highly pronounced at higher latitudes, whereas close to the equator they are much smaller. The

length of the day respectively of the night must be determined somehow. A signal is evoked which is the cue for the animal to induce the photoperiodic reaction.

We have seen before (chapter 12) that built-in annual clocks might also serve an animal as a reminder of the season. However, even in this case the annual rhythm has to be entrained to the time of the year. Otherwise it would quickly loose synchronization with the seasons. The cue used for this purpose is in most cases the photoperiod serving as a time of year-reference.

Photoreceptors are thus needed, and a photoperiodic time measuring system. This could be an hourglass type system, where the length of the night, for instance, leads to photoperiodic induction of reproduction if a certain critical length is reached or exceeded. However, it was found that the time measuring system uses a circadian clock[11].

As discussed in section 20.16, several models were proposed which can explain how the photoperiodic time measuring mechanism works. An external coincidence model could do the job, in which the circadian oscillator has to be illuminated at certain phases and kept dark in other phases if photoperiodic induction should occur. But an internal coincidence model with two oscillators driven independently by external cues connected with day-length[12] could also form the basis of the photoperiodic time measuring system. In more and more systems studied two oscillators have been found which compose the circadian system ([136], [669], see also recent results of SCN morning/evening oscillator, [692]).

The photoperiodic reactions of the Djungarian hamsters can be explained by both, an external and an internal coincidence model (see [506]). However, an internal coincidence model seems to be more appropriate (see figure 20.28 and [669], [1117], [136], *[506]*). In favor of it are results of experiments using light pulses of 0.5 hours duration, which were given every 23.0 to 25.3 hours to two different phenotypes: One of it follows a 9:15 hour short day, the other not ([1186]). Details of and differences between the external and internal coincidence model are given in section 20.16.

Photoperiodic center, perception and transduction of the photoperiodic signal

The circadian basis of photoperiodism is well established for hamsters, as shown before. But where is the external signal perceived and transferred to the center which serves as a 'clock for all seasons´ ([1163])? Where is this center located in mammals and how are the photoperiodic reactions realized? Is it the same center which governs circadian rhythms, namely the SCN? This has indeed be shown to be the case (reviewed by [1345]). If the SCN is destroyed, photoperiodic behavior is abolished. In the tau mutant which exhibits a 20 hour circadian period, the photoperiodic response is also altered. But it is 'normal´ if based on a 20 hour circadian period ([1425]).

The perception of the photoperiodic signal occurs in mammals via the retina of the eyes. Day-length is conveyed directly via the retinohypothalamic tract and indirectly via the geniculohypothalamic tract (that is, the same pathways as for the

[11] The circadian master clock of mammals is in the SCN. If it is destroyed, the circadian rhythms are gone, but also the photoperiodic reaction ([1286], [1423]).

[12] for instance one connected to the onset of light, the other to the onset of darkness

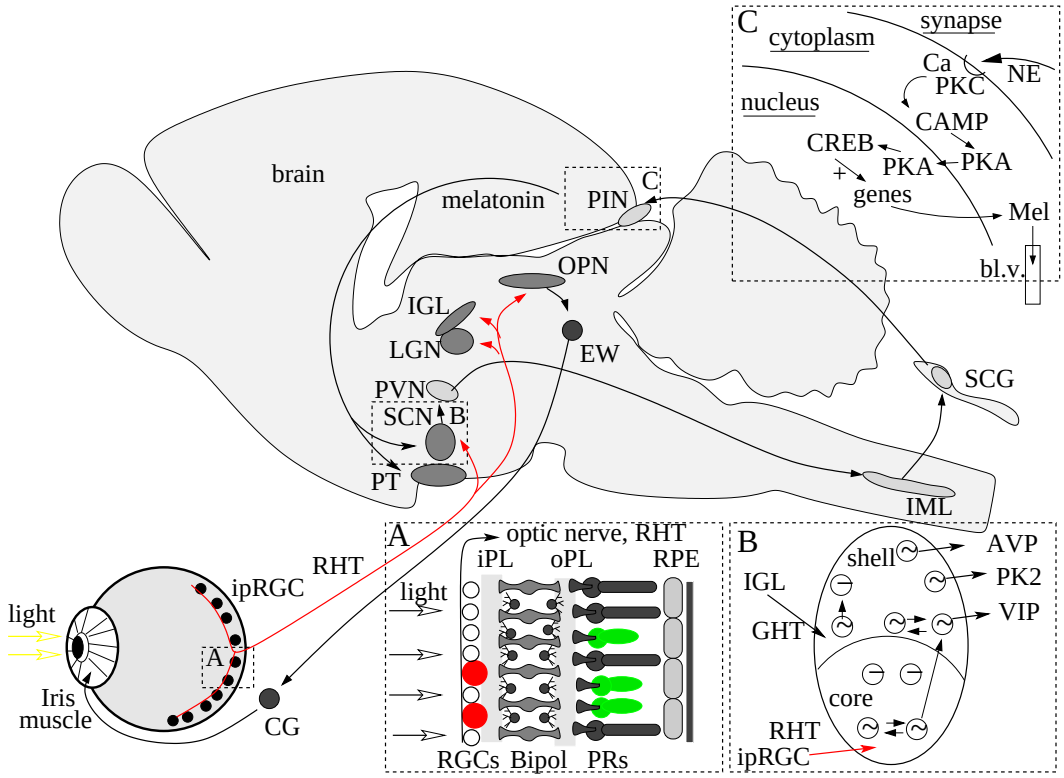

Figure 13.59: *Photoperiodic pathways in mammals: Day-length informations are perceived by the retina of the eye, transmitted to the SCN via a direct pathway, the RHT, and an indirect pathway, the IGT, to the SCN. After processing of the day-length information in the SCN neural signals (with GABA as a neurotransmitter) are sent to the paraventricular nucleus PVN. The PVN projects via preganglionic neurons of the sympathetic nervous system in the intermediolateral column (ILM) of the spinal cord (there are actually two parallel projections. One might serve to entrain the circadian rhythm in the pineal, the other to suppress the melatonin production in the pineal). Postganglionic cell bodies in the superior cervical ganglion (SCG) enervate the pineal. After* [1345]

light entrainment of the circadian system in the SCN) to the SCN. From here the day-length information is passed (after processing) to the pineal. The pineal transduces the photoperiodic information into melatonin as a readout signal. Melatonin is secreted only during the dark period. Via NAT activity melatonin is produced proportionally to the length of the daily dark period and secreted to the blood (and perhaps, or even more likely, to the cerebrospinal fluid) from where it reaches the target tissues. They ultimately regulate physiological changes and behavior ([951], [589]). The neural pathways from the SCN to the pineal are depicted in figure 13.59.

Targets of the photoperiodic center

If the SCN is indeed the center of photoperiodic control in addition to its pacemaker function for circadian rhythmicity, the pineal would be a subordinate center were melatonin is produced under the circadian control of the SCN and additionally inhibited directly by light. Further elements of the photoperiodic circuit are not well known.

Not known are also the neural substrates for circannual rhythms. Whereas SCN lesions abolish circadian rhythms and photoperiodic responses, the circannual rhythm of body weight in ground squirrels is not affected. Entrainment of the annual rhythm by light, its expression under low ambient temperatures and the timing of hibernation are, however, affected by SCN lesions.

The photoperiodic time, which has been encrypted by melatonin, must be decoded by melatonin responsive targets (‘melatonin readout´, [589]). The pars tuberalis in the hypothalamus is one of it. It contains the highest concentration of melatonin binding sites. The effects of melatonin on receptor expression and second messenger coupling have been studied. cAMP mediated pathways are important in the melatonin readout, but cAMP independent pathways are also involved. It is assumed that the expression of specific genes will be altered, most likely by affecting transcription factors. This will change the function of the melatonin-responsive tissue. The underlying molecular events are studied intensively and current results discussed by [589]. The issue is being complicated by photoperiodic history effects which have to be taken into account.

The most likely role of the pars tuberalis is to modulate prolactin secretion in lactotrophic tissue by producing a prolactin-releasing factor (‘tuberalin´). It has, however, not yet been isolated and identified.

Melatonin probably affects other sites in the brain to exert its effects on reproduction such as gonadotropin secretion, gonadal activities, sexual and maternal behavior ([951]).

13.5 Photoperiodism in the quail

The photoperiodic control of reproduction in mammals and birds has a number of common features. However, there are also differences which are partly due to the differences in biology in these classes of vertebrates (for instance adaptations to flight such as light weight, feathers, no milk in birds). In contrast to mammals birds do not require eyes for photoperiodic timing of reproduction and they do not use the melatonin signal as a measure of the photoperiodic situation.

In detail, the differences are found in the following:

- The annual rhythm of gonad size

shows a much higher amplitude during the course of the year as is the case in mammals. Gonadal size is several hundred fold larger in birds as compared to mammals (sheep: 2.5 fold, Syrian hamster: 3-5 fold). This is due to a more rigorous switch-off of the hypothalamus-pituitary-gonad axis, which leads to a greater regression. The advantage for birds is obvious, since for flight the body mass should be small. Bats as flying members of the mammals seem to have a similar tendency.

- The breeding season is more constraint as is the case with mammals. It is restricted to the time of food availability and therefore asymmetric to the photoperiod. That is, the termination of the breeding season occurs at longer photoperiods as its induction. Exceptions are *Columbiformes* which are milk feeders, and quails (see later).

- Molt is timed to the period of food abundance, occurring immediately after breeding. Molt is essential for high quality plumage and flight. It is timed by the photoperiod, but a longer breeding delays its onset. However, in this case the rate of molt is speeded up (with a poorer feather quality).

Most birds develop their gonads under long day conditions. The photoperiodic control of sexual activity depends on the length of the breading time. In birds of temperate latitudes with long breeding seasons, which do not migrate, the begin and end of the breeding season is controlled photoperiodically. In other birds of temperate zones the breeding season is photoperiodically induced and terminated by a negative feed back. In migrating birds of higher latitudes the breeding season is photoperiodically induced and terminated by a refractory period, in which they do not react photoperiodically (figure 13.60).

The quails belong to the latter group. Their photoperiodic reaction can be studied easily: The animals react already to a single long day. Instead of waiting, until the gonad development is visible, the concentration of the luteinising hormone (LH) in the blood can be used as an early hand[13]. Furthermore the animals can be kept easily and have a delicate taste. These are the reasons why quails have been used for numerous studies.

13.5.1 Systematics, habitat

Coturnix coturnix coturnix and *Coturnix coturnix japonica* belong to the chicken birds (*Galliformes*). This order consists of 7 families with 250 species. The family of pheasant birds (*Pasianidae*) contains 170 species, among them the quails. The quail is about 20 cm large, has a brownish protective color and is found in Eurasia from the Atlantic to Japan. In Tibet they are found up to a height of 3000 meter. It is also found in northwestern Africa, southern Africa and Madagascar. In central Europe the birds arrive in May and leave in August/September for northern- and central Africa, where they over-winter.

The behavior of the Japanese quail has been studied among others by [1011].

13.5.2 Photoperiodic control of reproduction

The reproduction of quails follows an endogenous annual rhythm. This rhythm is

[13] LH controls in vertebrates gonadal growth

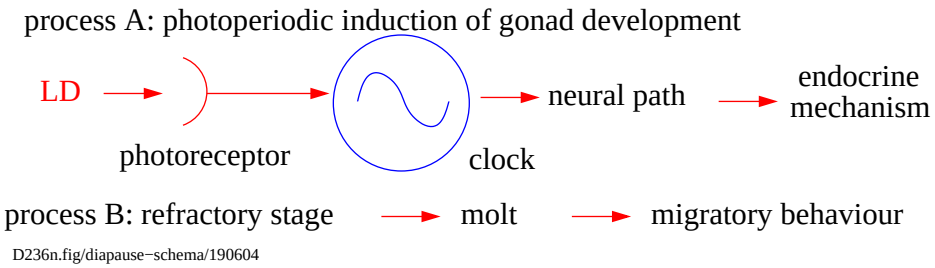

Figure 13.60: *Photoperiodic control of sexual activity in migrating birds of higher latitudes. The breeding season is photoperiodically induced (process A) and terminated by a refractory period, in which they do not react photoperiodically*

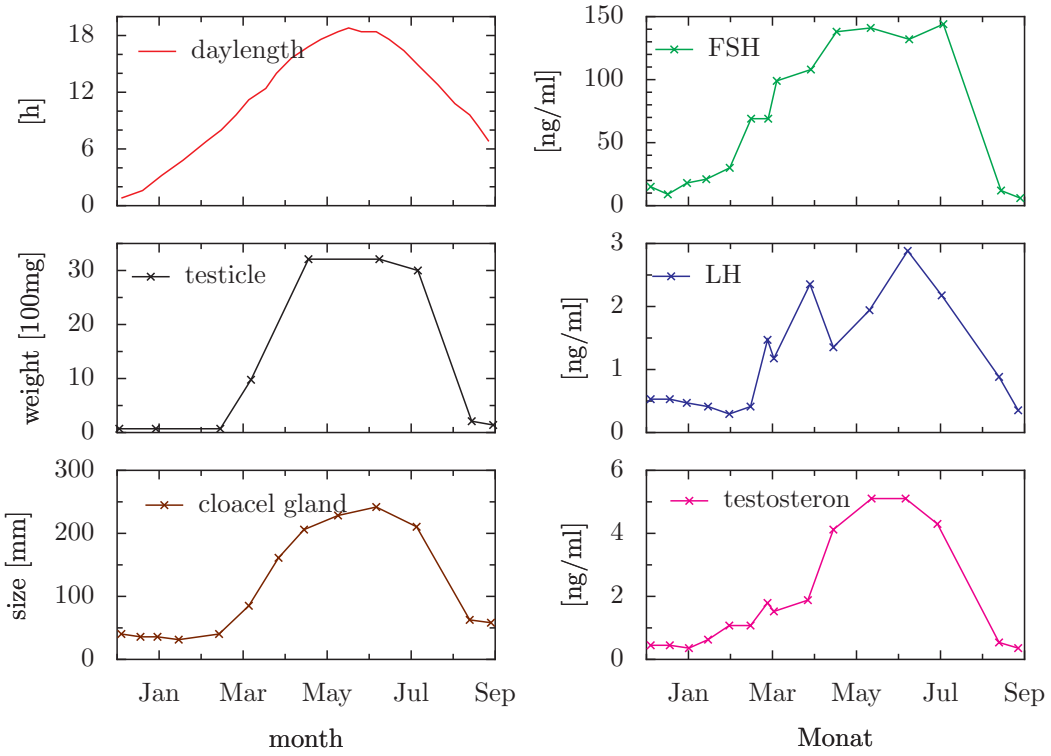

Figure 13.61: *Photoperiodic reactions in quails. Top left shows day-length as a function of time of year. Left center: Testis weight, left bottom size of cloakal gland, top right FSH, right center LH, and right bottom testosterone, all as function of time of year*. According to [443]

synchronized to the season by photoperiodic means. The photoperiodic effect can be followed by using the following 'hands' (figure 13.61):

- body weight,
- size of the cloakal glands,
- testicle volume and number of spermatozoa
- activity
- diameter of the ovary follicles
- luteinising hormone LH
- prolactin
- egg production

The size of the cloakal glands can be measured easily from outside. The volume of the testicles can be judged by palpation. The activity of the animals was measured with wiggle cages. LH can be determined by radioimmunological methods in the μl-range in the blood. Prolactin controls the fat deposition. Fat is important for migration. Of practical significance is the rate of egg production. It depends on day-length, light intensity and cycle length.

As a hand of the effect of a photoperiodic treatment LH is often used. Its concentration increases as a consequence of the long day-treatment. Already 22 hours after onset of light it reaches a maximum, which stays on for three weeks. Are there even earlier signs of the long day effect than LH? The GnRH of the hypothalamus triggers LH secretion in the hypophysis. It was therefore studied, when after the photoperiodic induction GnRH secretion occurs. The time difference between the releasing hormone and the LH was, however, small. C-fos on the other hand is already produced 18 hours after onset of the inducing long day, that is 4 hours before the LH-increase.

Quails become reproductive from a certain critical day-length onward under long day conditions. After a certain time they become photoperiodically refractory, even under prevailing long day. They need, however, short day, in order to be able to react photoperiodically to a long day again.[14] Short day can be replaced by a change between a short period of high and a long period of low temperatures.

Japanese quails were kept under natural temperature- and light conditions and the LH concentration was determined in the blood weekly. LH concentration increases, if the light period in spring exceeds 12 hours. In September the LH concentration decreases in spite of long day (14:10 hours of a light dark cycle) and the gonads regress ([1521]). A circadian and an annual rhythm seems to play a role. This becomes apparent, if the quails are kept from birth onward for 5 years at 20^0C in a 12:12 hour light dark cycle. In the first year the animals become fat after the first post juvenile molt. They undergo a period of increased nocturnal activity. In nature they would migrate during this time. From the fourth month onward sexual development starts. This is followed by the second post-juvenile molt and afterward for 6 months the time of reproduction. These events were repeating in the next three years (figure 13.62). In nature the sexual development depends on abiotic environmental factors and on social factors ([538]). The

[14] If the birds are exposed to a week of short day, they are not yet sensitive to a long day. With two weeks of short day they are somewhat, with three weeks they are strongly responsive and with 5 weeks all animals react. We are thus dealing with a graded and not an all-or-non-reaction ([444]).

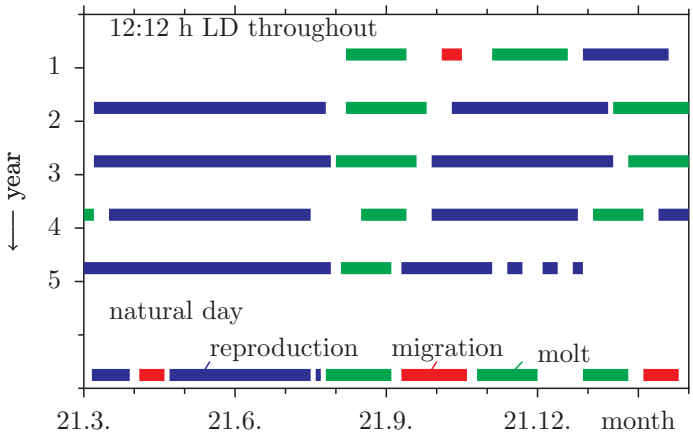

Figure 13.62: *Annual rhythm of the European quail during five years under artificial LD 12:12h throughout. Top: In the first year (born August) the quail would after a fattening period and after post-juvenile molt (green bar) migrate (nocturnal migratory restlessness in the tenth week, red bar). Reproduction (blue) is followed by second juvenile molt (green bar to the right). In the following years reproduction (blue), prenuptial molt green), reproduction (blue) and post-nuptial molt (green). In the following years reproduction, pre-nuptiale molt, reproduction, post-nuptiale molt.*
Under natural conditions in adults over one year old the post nuptial molt follows sexual regression (lowest row, end of July or early August, green). In 60% this post nuptial molt (green) consists of two phases, with a fattening phase between the molt phases, and lasts about 4.5-5 months. Four to five weeks of nocturnal restlessness follow (migration, red). After [538]

molt sequence may vary considerably, but the following can be taken as a reference: The first phase of the post juvenile molt lasts about four weeks. Four to five weeks of nocturnal restlessness follow. The second post-juvenile molt resumes for four to five weeks. At the end of December or early January the prenuptial molt begins and lasts for eight plus/minus four weeks depending on the sexual development. In adults over one year old the events are shown in figure 13.62, last row.

13.5.3 'Hardware'

The 'hardware' for the photoperiodic reactions in the quail as well as in other vertebrates is not well understood so far. A current model for mammals and birds is shown in figure 13.63. In mammals light is perceived in the retina of the eyes and affects the photoperiodic time measuring system via the SCN. According to the photoperiodic situation in the environment the function of the gonads is affected via a number of secondary processes, which are triggered by the photoperiodic signals. The pathways of these signals are complicated and different in mammals and birds. The interactions ('the play´) of the different players (eyes, SCN, pineal, gonads) are indicated in figure 13.64 for both, mammals (black) and birds (red).

Receptors of the photoperiodically effective light

In mammals the retinal elements of the eyes perceive the photoperiodically active light and transmit it further. Apparently the same light receiving elements are used which see the visual environment. A house sparrow on the other hand can be synchronized without eyes and without pineal by a light dark cycle ([997]). Blind quails[15] are still able to synchronize their activity to the light-dark-cycle and to react photoperiodically. Apparently for both tasks extra-retinal photoreceptors can be used. This seems to be valid for all birds, for different fish, amphibian and reptiles ([836]). In these vertebrates the brain is astonishingly well permeable for light. Photoreceptors in the brain might be able to receive the light conditions of the environment.[16]

The pineal, the parapineal organ, and photoreceptors at the brain ventricles are candidates. The pineal does not seem to play a role or only a minor one in the photoperiodic reaction of birds. If a pineal organ of a quail is locally exposed to a long day, gonadal growth is *not* stimulated ([655]). Pinealectomy in otherwise intact and also in blind quails does not affect the photoperiodic reaction ([1388]). If, however, the brain stem is illuminated via fiber optics, long day does have a photoperiodic effect ([1109], [1597]). Neurons with club-like appendixes, which protrude into the cerebrospinal fluid of the brain ventricles, could be the photoreceptors ([1522], [1402], [1514]). Figure 13.64 shows the different photoreceptors and their connections to the SCNs, the circadian outputs and feedbacks of these outputs to the circadian system.

The action spectrum of photoperiodically effective light has a maximum at 500nm ([440]). The sensitivity at 500 nm

[15] Formoguanamine HCl was used to degenerate the retina

[16] The eyes play, however, also a role. If they are covered to prevent light from being absorbed, the rhythm falls apart into two components, one (O_s) with a period length of 22.7 hours and another (O_l) with a period length of 26.3 hours. If the eyes are destroyed, the animals become arrhythmic ([1497]).

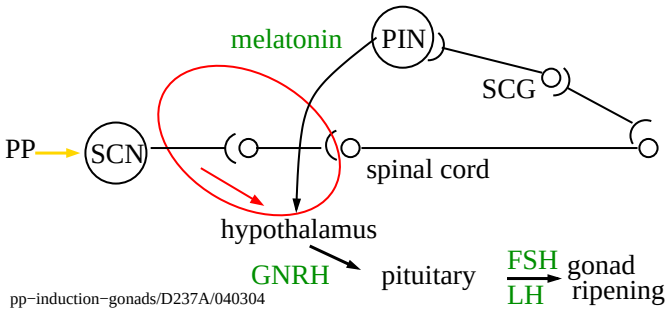

Figure 13.63: *Control of gonadal function by photoperiod (PP, yellow arrow) in mammals (black and green). Daylength signalled from the retina of the eyes to the suprachiasmatic nucleus (SCN). Neuronal signals transferred through spinal cord and upper cervical ganglion (SCG) to pineal (PIN), where melatonin (green) is produced and secreted. Night length determines length of melatonin secretion and thus conveys photoperiod to hypothalamus. Neurosecretory cells in hypothalamus produce gonadotropine releasing hormone (GnRH, a dekapeptide, green). It reaches anterior hypophysis via portal vein. In hypophysis luteinizing hormon (LH, green) and follicle stimulating hormone (FSH, green) are produced and secreted to gonads. They induce gonads to ripen and to be active.* ***Red****: In birds photoperiodic signals are perceived extraretinal and affect the hypothalamus. Gonadal ripening induced via hypophysis which lasts in quails three to four weeks. Afterward animals become refractory (tyrosin dependent) and gonads are inactive. The mammalian pathway through the spinal cord and pineal is not involved in birds*

was $2.8 * 10^{12} \mu E/cm^2 sec$.[17] The photoreceptor is probably rhodopsin with an absorption maximum at 492 nm.

The circadian system is used for photoperiodic time measurement

After the light dark cycle was perceived by a receptor, the organism has to decide, which day-length and/or night length prevails. Quite early [180] proposed, that for this purpose the circadian system is used. There are indications, that the time measurement for the photoperiodic control of the sexual activity in birds is also exerted by a circadian clock. If, for instance white-crowned sparrows are transferred from short day-conditions into a long dark period of five days and at different times individual groups illuminated for 8 hours, the photoperiodic induction of the gonad development is triggered at certain phases only (figure 13.65). Light must hit the animals at appropriate times in the circadian cycle, in order to be photoperiodically effective.

This was shown to be valid also in quails. However, the circadian fluctuations were less pronounced (figure 13.66). The time span in which light is photoperiodically inductive, is termed photoinductive phase Φ_i. It lies in the quail 12 to 13 hours after the onset of light and is about 4 to 6 hours long (figure 13.67). The circadian system of the quail is, however, relatively weak expressed. There are indications that other circadian rhythms such as the locomotor activity and that of melatonin in the blood are no useful hands of the circadian modulation of the photope-

[17] $1 \mu E/cm^2 sec$ corresponds to $1 * 10^{17}$ photons

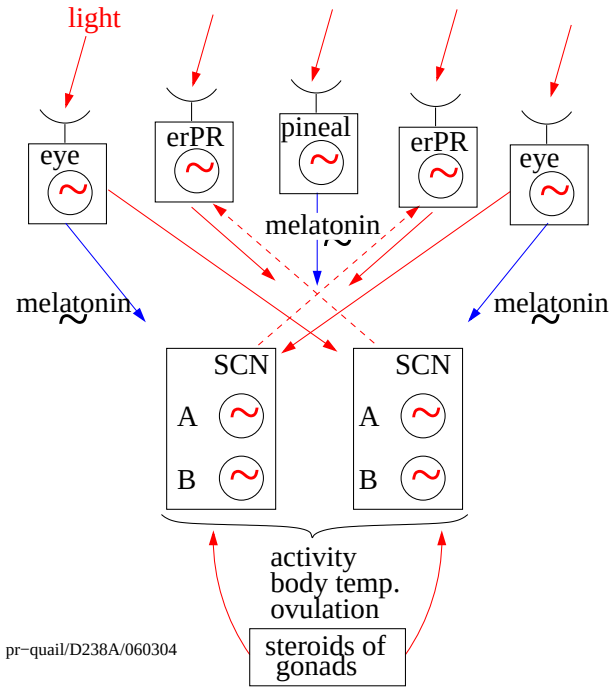

Figure 13.64: *The circadian system of the Japanese quail consists of ocular oscillators (eye) and the paired SCNs with two populations of oscillator cells (A and B). One subset of oscillators (A) is coupled to the ocular oscillators via the retinohypothalamic tract (RHT) and via hormonal output (melatonin) from the eyes. The other subset (B) is under feedback control from reproductive hormones (gonadal steroids, perhaps via hormone-sensitive neurons). The pineal gland is not an autonomous oscillator. It is driven by the light-dark cycles or by neural inputs from the SCN and secretes melatonin during the dark periods. The circadian system drives among other events locomotor activity, body temperature and via the HPG-axis (hypothalamus/pituitary/gonads) reproduction. The female gonadal steroid output feeds back to the SCN. Light affects the system (red arrows) via photoreceptors in the pineal, the eyes and in extraretinal structures. After and [1615]*

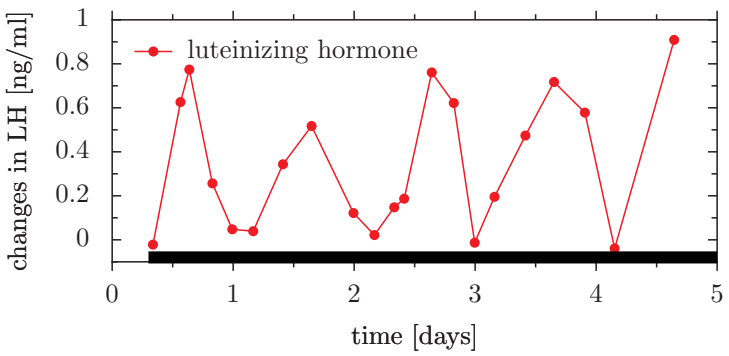

Figure 13.65: *White-crowned sparrows (*Zonotrichia leucophrys gambelii*) were transferred from short day-conditions (8:16 hours LD) into a long dark period (dark area) of five days. At different times individual groups (6 to 16 birds) were illuminated for 8 hours. As a hand of the photoperiodic induction the concentration of the LH in the blood was measured (y-axis). The photoperiodic induction of LH production was triggered only at certain phases, which repeated them self in a circadian rhythm. After [442]*

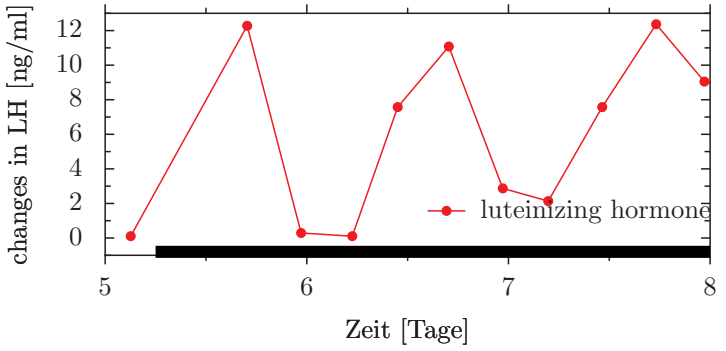

Figure 13.66: *Quails were transferred from short day conditions (6:18 hours LD) into a long dark period (dark area) of three days. At different times individual groups were illuminated for 10 hours. As a hand of the photoperiodic induction the concentration of the LH in the blood was measured. The photoperiodic induction of the LH-production occurred at certain times only, which re-occurred in a circadian rhythm. After [439]*

riodic reaction. According to these findings the circadian system of the quail consists of several oscillators. One of it controls the photoperiodic induction of the LH-production, which finally leads to gonadal development. More in the following.

Hormonal consequences of the long day

In the hypophysis Lh and FSH is secreted due to the gonadotrophic releasing hormone (GnRH).

The HPG axis mediates the photoperiodic information "long day" via the hypophysis to the pituitary and to the gonads. As a result, breeding begins. The breeding season is, however, asymmetrical in respect to the photoperiod: During winter and early spring the amount of gonadotropin releasing hormone GnRH is low and the gonads immature. In late spring long days stimulate GnRH release, In the hypophysis the gonadotrophic releasing hormone (GnRH) triggers LH and FSH secretion, gonads mature and the birds start to breed. While still under longday conditions, breeding is terminated and molt induced (see figure 13.68). Later photorefractoriness dissipates and GnRH concentration increases again. This stage corresponds to pre-pubertal conditions again.

The following areas of the hypothalamus are important for the photoperiodic induction of the GnT-secretion: The ventral and posterior part of the infundibular nucleus and the preoptic area. The hypophysis influences the target organs of the reproductive axis: The LH secretion is triggered by LHRH. The latter has to be provided episodically in an hourly measure. If provided in a shorter measure (10

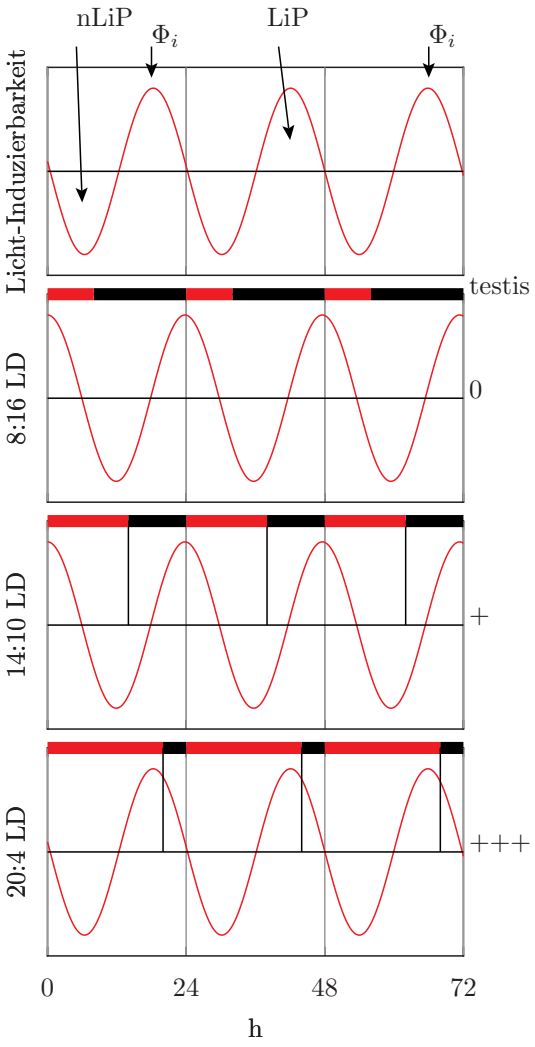

Figure 13.67: *Duration and position of the photo-inductive phase Φ_i in the quail. The animals were kept in a 6:18 hour light dark cycle. Different animals were treated at various times during the dark period with a four hour light pulse. The photoperiodic induction was measured by determining the LH-concentration in the blood after a 12 hour dark period. After [439]*

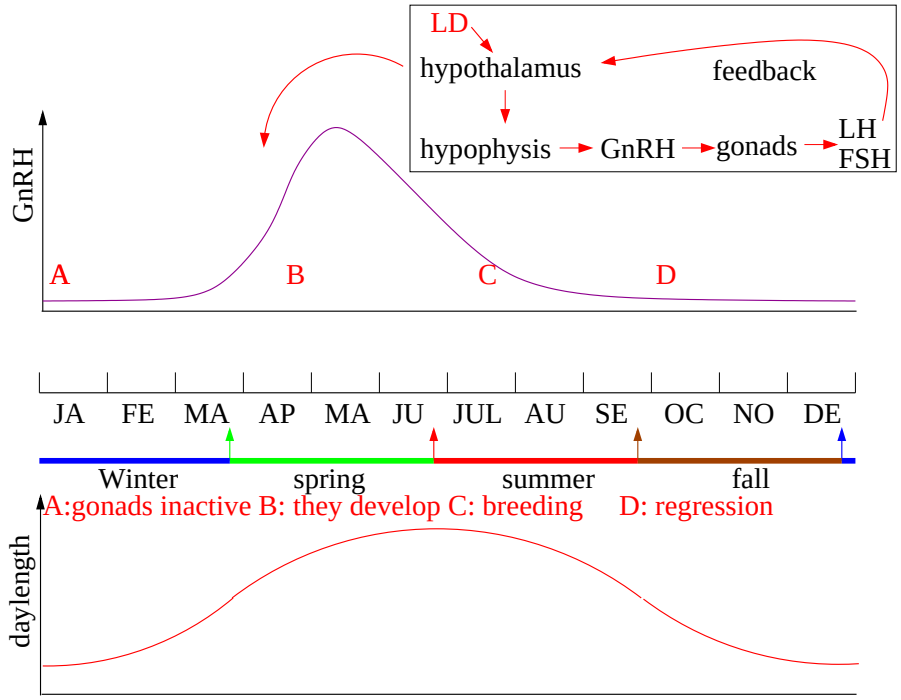

Figure 13.68: Asymmetric breeding season in quail: A. During winter and early spring the amount of gonadotropin releasing hormone GnRH is low and the gonads immature. B. In late spring long days stimulate GnRH release, gonads mature and the birds start to breed. C. While still under longday conditions GnRH production is terminated, refractoriness induced and the gonads regress. D. Photorefractoriness dissipates. As soon as longdays begin, the GnRH concentration increases again. B and C constrain time of breeding. Both, B and C are induced by long day, but the switch off of GnRH production during C occurs also under longdays of the summer

minutes), only little LH is secreted, if provided continuously, none is secreted. Between the different targets are feedbacks, for instance between the gonads and the hypophysis.

To see, what happens, if quails are transferred from short day into long day, fos-like proteins[18] in the neurons from the eye to the brain were followed. After this transfer into long day the number of neurons with fos-like proteins increases. They lead via the median eminence with the infundibular nucleus to the basal tuberal hypothalamus. Furthermore the density of melatonin-receptors in the nuclei of tectofugal pathways changes after the transfer. They are important for visual patterns, light intensity, localization and orientation.

Melatonin and the pineal

Signals of the SCN are transferred to the pineal. The melatonin of the pineal is distributed in the body via the blood system. In this way it also reaches the Harderian gland which possesses many melatonin receptors, and the gonads. But the cardiopulmonary system (heart, lung) has also numerous melatonin receptors. It is responsible for the different energy demand of peripheric tissue under various photoperiods. Melatonin reaches also the hypophysis via the hypothalamus.

Cultures of the pineal organ of the quail show only a weak melatonin rhythm or even arrhythmia in continuous darkness. In that they differ from other birds ([103] und [749]). Apparently their circadian organization is at variance from that of other birds.

What is the photoperiodic signal in birds? In mammals it was the duration of the melatonin, with which the length of the dark period was measured. In birds it is not the duration. Melatonin seems to be only a Zeitgeber for the circadian rhythm. It is coupled to the external Zeitgeber (the photoperiod). Birds possess accordingly also a melatonin-calendar, but they do not use it to measure the photoperiod (at least not for the photoperiodic control of reproduction). Instead they decode neuronal SCN-signals, which reflect the photoperiod ([441]).

Melatonin is also found in the retina of the eyes[19]. During the light period the concentration is low, during the dark period high. Under continuous darkness the melatonin concentration fluctuates for two more days in a circadian way. Under continuous light no melatonin rhythm is found. The melatonin-rhythm in the eye is already observable in quails during hedging. The melatonin rhythm can be synchronized in both eyes independently from each other. This indicates, that the oscillator as well as the photoreceptor for the melatonin-rhythm is in each individual eye and that the melatonin rhythms of the two eyes are not coupled with each others. The sympathetic innervation of the eyes from the upper cervical ganglion and from the isthmo-optic nucleus of the midbrain are without influence on the melatonin rhythm in the eye.

Melatonin shortens the activity of the animals. At low melatonin concentration the activity increases and it begins earlier. Low melatonin-concentration is a prerequisite for the onset of reproduction.

In the Indian jungle-quail *Perdicula asiatica* not only the pineal but also the Harderian

[18] immediate early genes

[19] The retina as well as the pinealocytes develop during the ontogeny from the same brain regions.

glands react photoperiodically ([336]). The latter contain also numerous melatonin receptors. The weight of the pineal, of the Harderian gland and of the ovaries fluctuates in an annual rhythm with a maximum at the end of May for the Harderian gland and the ovaries and a minimum for the pineal. In May the weather is especially favorable. An inverse relationship seems thus to exist between the weight of the Harderian gland and ovaries on the one side and the pineal on the other. Parallel to it run the melatonin and the 17-β-estradiol values and the porphyrin-values in the Harderian gland.

13.5.4 What happens after the photoperiodic induction?

LH serves as a hand of the effect of the photoperiodic treatment. Its concentration increases as a consequence of a longday treatment. 22 hours after onset of light it reaches a maximal value, which is maintained for three weeks. This 'carry over effect' is known also from photoperiodically induced plants: An induced leaf maintains this inductive stage even under non-inductive conditions. It can be transferred to non-induced plants by pruning.

Are there earlier hands than LH? The GnRH of the hypothalamus induces the hypophysis to secret LH. It was therefore investigated, at what time after the photoperiodic induction GnRH occurs. There was, however, no major difference in time of occurrance between the releasing hormon and the LH. The cfos is, however, formed already 18 hours after onset of the inducing longday, that is 4 hours before the LH-increase.

13.5.5 Circadian clock and photoperiodic time measurement: Internal coincidence

In mammals and birds it was assumed, that an external coincidence model explains the photoperiodic reactions (see under special topics section 20). In quails however the concentration of the LH increases always independently of the length of the light period of the long day at the 20th hour. Nanda-Hamner-experiments give results, which are not in accordance with the expectations according to the external coincidence model. It is likely, that the circadian rhythm of the quail is only slightly self exciting and damps out quickly ([441]). On the other hand an internal coincidence seems to be important. It was shown, that the neurotransmitter serotonin and dopamin fluctuate both in a circadian way, but not in phase with each other. If they are injected either with an 8 or with a 12 hour interval to each other, gonadal growth is in the first case suppressed even under longday conditions. In the second case sexual maturation, spermatogenesis and egg production occur premature. This speaks in favor of an internal coincidence principle ([336]).

Several circadian clocks may be present in the quail: The photoperiodic sensitivity fluctuates in a circadian way, but this photoperiodic response rhythm (PRR) is driven by a circadian oscillator which is different from the one driving the locomotor activity of the animals. The activity can therefore not be used as a hand for the PRR. A similar result was obtained from the fly *Calliphora* ([1321]).

13.6 Photoperiodism in humans and other primates?

Since the anatomical and functional basis of the photoperiodic system of mammals is found also in monkeys and primates, it was obvious to search for photoperiodic reactions among this group. Evidences and implications are reported by [1539].

Seasonal breeding is widespread among primates. Among prosimians, old- and new world monkeys short-day and long-day breeders, but also nonseasonal breeders have been found. Seasonal breeding depends on diet, latitude and body size. Birth occurs usually shortly before the time, at which much food is available. However, photoperiodism and not food availability seems to be the proximate factor which is used in the animals to time birth ([900]). Rhesus monkeys are short-day breeders. Females show seasonal patterns in ovulation, mating behavior, conception, body weight and levels of sexual hormones. In males the body weight, fat levels and testicular function change also with season. They are apparently under control of an annual rhythm, but synchronized by the photoperiod. Circannual cycles control also reproduction in Squirrel monkeys and photoperiod entrains the rhythm to the adequate season.

Human reproduction is also subject to seasonal variation, and this is more obvious from data of conceptions from historical periods (see figure 13.69 and [1539] for discussion). Furthermore, women who were born at certain times of the year vary more in rates of conception during the course of the year than other women. There may be individuals in the population who are more responsive to seasonal effects than others (see [166] and the commentary of [1244] with a reply of [167]). Reproduction in humans may be stimulated by the lengthening of the day during spring. The average temperature which changes with season may also play a role. However, environmental light intensity seems to be the major variable, and photoperiod is subordinate ([272]).

The seasonal affective disorder (SAD) has been mentioned already (subsubsection 2.12.2). It may be related to photoperiodic effects.

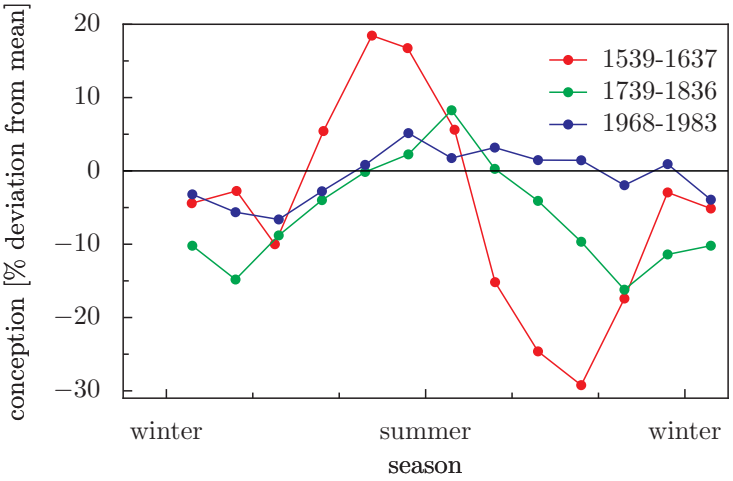

Figure 13.69: *Annual variation of conception in humans during three historical periods in England. Deviations from the mean. Note that amplitude decreases from the elder records (1539-1637, red) to the more recent (1968-1983, blue). The seasonal effect is much reduced in the last period shown, where artificial illumination in houses is standard practice. After [1539]*

14 Clocks of *Drosophila*: hands, localization, control

Drosophila *exhibits a number of different rhythms. The circadian eclosion of the adults out of the pupal case and the locomotor activity rhythm of the flies are the most studied one. Both will be the subject of this chapter. The eclosion rhythm can be recorded only in a population of pupated flies: It is an event which happens only once during the development of a fly. At a certain time during the development a clock opens a time gate. It is only in this gate when a fly can eclose. We will see why the animal uses such a gate. We will get to know the different properties of the eclosion clock. Light is perceived via extraretinal photoreceptors and synchronizes the eclosion rhythm to the light-dark cycle of the day.*
The circadian locomotor activity is also synchronized by photoreceptors to the light-dark cycle. Under constant temperature- and light conditions the rhythm continues to run. In contrast to the eclosion rhythm, its period length is, however, in the individual flies generally not exactly 24 hours, but shorter or longer. The period depends furthermore on the light intensity. Different mutants were isolated with an altered circadian rhythm. Genetic and molecular biological methods were used, to find out how the circadian mechanisms function. Certain neurons in the brain are the centers, which control the locomotor activity.
How the eclosion rhythm and the locomotor activity is recorded and analyzed is also described.

In *Drosophila* several circadian rhythms were observed and studied. Among them are also circadian controls of the behavior such as timing of eclosion of flies from the puparium and locomotor activity of the adults. [87] put forward the idea, that behavior can be traced to the action of specific genes and proposed to look for mutants with altered behavior. This was done by his student Konopka for the circadian control of eclosion ([813]) and resulted in the detection of three mutants per^s, per^l and per^0, which exhibited a shorter, longer or no rhythm as compared to the wild type. It turned out that these mutants were all point mutations in the per gene and that the gene is together with other clock genes essential for the functioning of the circadian clock mechanism (see section 14.2.5). Let us first have a closer look at the eclosion rhythm.

14.1 Eclosion of *Drosophila*: A population rhythm

The fruit fly *Drosophila* is often found on fermentizing fruits. The males court the females in a characteristic way. After copulation the females search for a suitable place to deposit their eggs. The larvae live on yeast cells, grow and molt three times. At the end of the fourth larval stage the animals crawl out of the food and try to find dryer localities. They form a pupal case (puparium) and change from a larva into a fly (metamorphosis). The fly opens a preformed lid (operculum) of the puparium and makes its way out (figure 14.2).

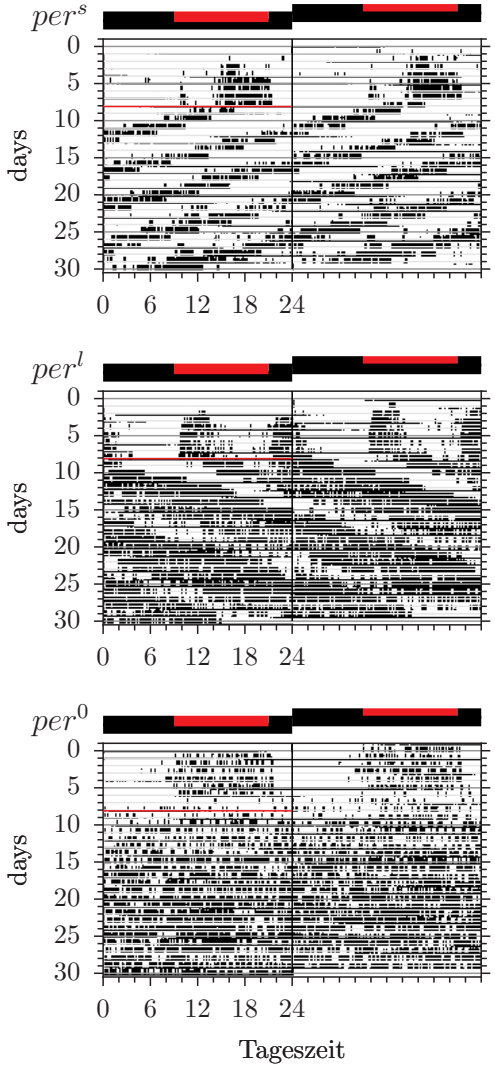

Figure 14.1: *Actograms of wild type (top left) of per^s (top right), per^l (bottom left) and per^0 (bottom right) of* Drosophila melanogaster *flies. Doubleplot (2*24 hours) under 12:12 hours LD in the first seven days and continuous darkness for 21 days subsequently. Bars on top indicate light program (black: darkness). Period length of wild type 23.3 hours, of per^s 19 hours (activity pattern runs to the left), of per^l 29 hours (activity pattern runs to the right). The arhythmic mutant is still entrained to the LD cycle, but lacks the biphasic pattern of the other mutants and the wild type in LD. After [603]*

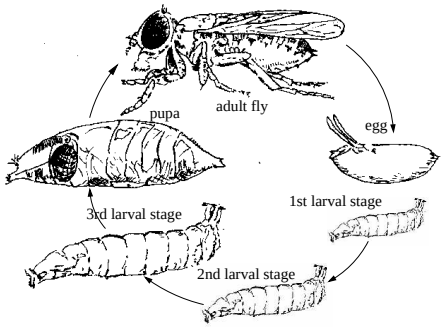

Figure 14.2: *Life cycle of the fruit fly* Drosophila. *Female fly (top) lays eggs (one shown right), from which larvae hatch. They undergo three stages with moltings in between. The third stage (left bottom) pupates in a puparium (left center) in which metamorphosis to the fly takes place. After [481]*

We will now have a closer look upon eclosion using *Drosophila pseudoobscura* as an example. In this species from the southern parts of the United States the time course of eclosion has been studied mainly by Pittendrigh and his coworker ([1158], [1575]). If we observe many pupae, eclosion from the pupal case is not uniformly distributed over the day. Instead it occurs in the early morning shortly after sunrise. Each fly can of course eclose only once. It uses a gate of about 4 hours. If it is not yet ready to eclose, it will wait for eclosion until the next gate on the following day. Figure 14.3 shows in the central curve the number of animals eclosed per hour during a week at 21^{0}C. Each day we observe in the first four hours after onset of light high eclosion rates. During the afternoon and night no flies eclose.

Two question pose themselves:

- Why do the animals eclose only during a gate?

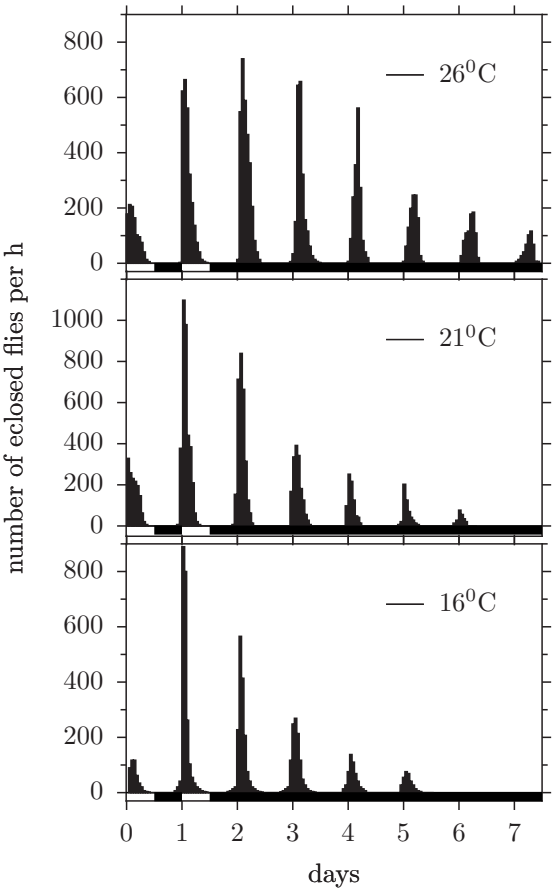

Figure 14.3: *Number of* Drosophila *-flies eclosed per hour during a week at three different temperatures. The cultures were first kept for two days in a 12:12 hour light-dark cycle. In the first four hours after onset of light high eclosion rates are observed, during the afternoon and night no flies eclose. Afterward eclosion was recorded in weak continuous red light. The flies continue to eclose in a circadian rhythm. The period length is at all three temperatures almost the same and shows thus the temperature compensation of the eclosion rhythm. After [949]*

- How do the animals know, that a gate is open?

14.1.1 Time windows are determined by a circadian clock

The first question is not easy to answer. We will come back to it later. As to the second question we can put forward a few hypotheses and try to test them experimentally.

Hypothesis 1: With the sun rising in the morning temperature will increase. This might cause the animals to eclose (provided they are far enough developed). We test this hypothesis in a room with constant temperature. The sunlight is replaced by a white fluorescence tube. It is switched on each day by a timer for 12 hours and afterward switched off for 12 hours. Result: In spite of the constant temperature the animals continue to eclose in the first hours after onset of light. The increased eclosion rate does therefore not depend on an increased temperature.

Hypothesis 2: The light in the morning is responsible for the increased eclosion rate. This hypothesis is also testable. Pupae of different age which will eclose in the following days, which have been kept in 12:12 hour light-dark cycles, are transferred in weak red light. It will not be switched off for the whole observation period. For the animals red light is like darkness (safe light). We are, however, able to observe eclosion of the animals in this light. To our surprise many flies eclose again in a four hour gate shortly after the time at which normally the white light would have started. And if we continue to observe for another day, we find eclosion maxima to occur in a 24 hour measure. It is as the animals are able to use the gate for eclosion even without external time cues

(Zeitgeber).

Hypothesis 3: The animals are able to use environmental factors as Zeitgeber, which in our chamber occur in a 24 hour measure in spite of the constant temperature and continuous red light. As a test we offer the pupae before eclosion an inverted light-dark cycle: The 12 hours light period is now during the night time, the dark period during the day time. We find, that the animals eclose preferentially in the first hours after onset of the light period, that is at night outside the room. If we offer after the inverted illumination continuous red light, the animals eclose 24 hours after the increased eclosion rate of the inverted light-dark cycles. Would external Zeitgeber be responsible for the four hour eclosion maximum, they should induce eclosion under continuous red light at the normal time. This shows, that a light-dark cycle is a Zeitgeber for eclosion: The rhythmic eclosion can be shifted to other times of the day. But under constant conditions this rhythm continues without being affected by possible daily time cues in the external environment.

From this we must conclude: The *Drosophila*-flies possess internal clocks, which allow eclosion to occur at certain gates only in a 24-hours measure. These clocks can be shifted by the light-dark cycle.

14.1.2 Why do the animals eclose in gates only?

We will try now to answer the first question: Why do the *Drosophila*-flies eclose only in a gate? For this purpose we have to look at the biology of the animals. *Drosophila pseudoobscura* lives in arid areas in the southern United States. Before pupation occurs, the larvae crawl at the end of the fourth larval stage into the soil. In a depth were no light can be seen and no temperature differences are found the metamorphosis to a fly occurs. It will be completed after 7 days. The eclosed flies will crawl to the surface of the soil. In the first hours the cuticle hardens and the animals are able to fly.

The cuticle has for some time not yet hardened and is water permeable. Eclosion has therefore to occur at a time at which the humidity of the air is high. This is the case in the early morning. The inner clock determines the gate for eclosion at the end of metamorphosis in such a way that the animals reach the surface at the most favorable time of the day. Since the pupae are about 7 days submerged in the soil the clock has to be quite precise and has to open the gate exactly every 24 hours. The eclosion clock is one of the few cases in which the measure is exactly 24 hours. Most daily rhythms run under constant conditions not exactly in a 24-hour cycle. They are therefore called circadian clocks (see section 18.2).

14.1.3 Properties of the eclosion clock

Although the eclosion rhythm has a rather precise 24-hours measure, there is an indication that we are dealing with an internal clock (in the case of a period length of exactly 24 hours there is always the suspicion, that a hidden Zeitgeber drives the rhythm): Mutants of *Drosophila* are available the clock of which runs faster (per^s), or slower (per^l) ([813]). It is expressed in a shorter than 24 hours period (per^s) or a longer than 24 hours period (per^l) of the circadian eclosion rhythm. This indicates, that eclosion is indeed controlled by an internal clock.

There are, by the way, mutants, which eclose earlier than the wild-type (*early*) or later (*late*), although the period length of the eclosion rhythm is normal under constant conditions ([1162]). Apparently the coupling of the clock to the light-dark cycle has been changed by the mutation.

Clocks need not only to have a clockwork and the possibility to set it, but also another important property: They should not run faster at higher temperatures and slower at lower temperatures. This is indeed the case in the eclosion rhythm (figure 14.3). In the physiological temperature range rhythmic eclosion of the flies is, independent of the environmental temperature, always around 24 hours ([1158]). In spite of it temperature can have an influence on eclosion. If the pupae are treated in continuous red light with a 12:12 hour temperature cycle (of for instance $25^0/20^0$C), the eclosion rhythm can be synchronized by it (figure 14.4). Even a single temperature pulse (for instance of 3 hours duration) can phase shift the rhythm under constant conditions.

The same is true for a single light pulse (figure 14.5). Its effect depends on the strength of the pulse and the time of its application. The effect can be seen from phase response curves (figure 14.6).

At subjective midnight the shift of the rhythm is strongest, if the light pulse is strong. Winfree (summary in [1574]) could show that a special light pulse administered at a certain time during the cycle can stop the rhythmic eclosion altogether. The animals eclose now randomly. This special time is the subjective midnight. The light pulse has to be just strong enough that the reaction lies between a strong and a weak response (see figure 14.7). Arrhythmicity could be induced by special treatments also in other circadian rhythms

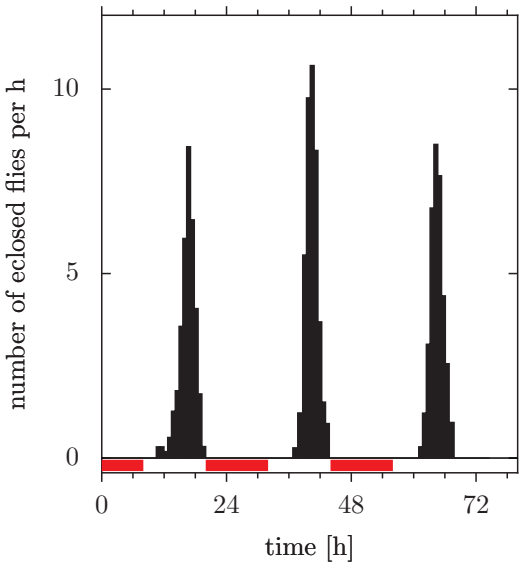

Figure 14.4: *A 12:12 hour temperature change from* $28^0(red)/20^0C$ *synchronizes the eclosion rhythm of* Drosophila pseudoobscura. *The larvae and pupae were kept under continuous red light. After [1612]*

([1574], [1572]).

14.1.4 Photoreceptors for the synchronization of the eclosion rhythm

Since eclosion rhythm can be shifted by a light-dark cycle and by a single light pulse, light receptors must exist which transfer the light signal to the oscillator controlling eclosion. How do we find out which one are used?

The standard method is to obtain an action spectrum and compare it with known absorption spectra of different photopigments. For an action spectrum colored light is applied to the organism and the effect ('action') measured as a function of wavelength. If coloured light of a certain wavelength is inactive, no effect will be observed. The more effective a cer-

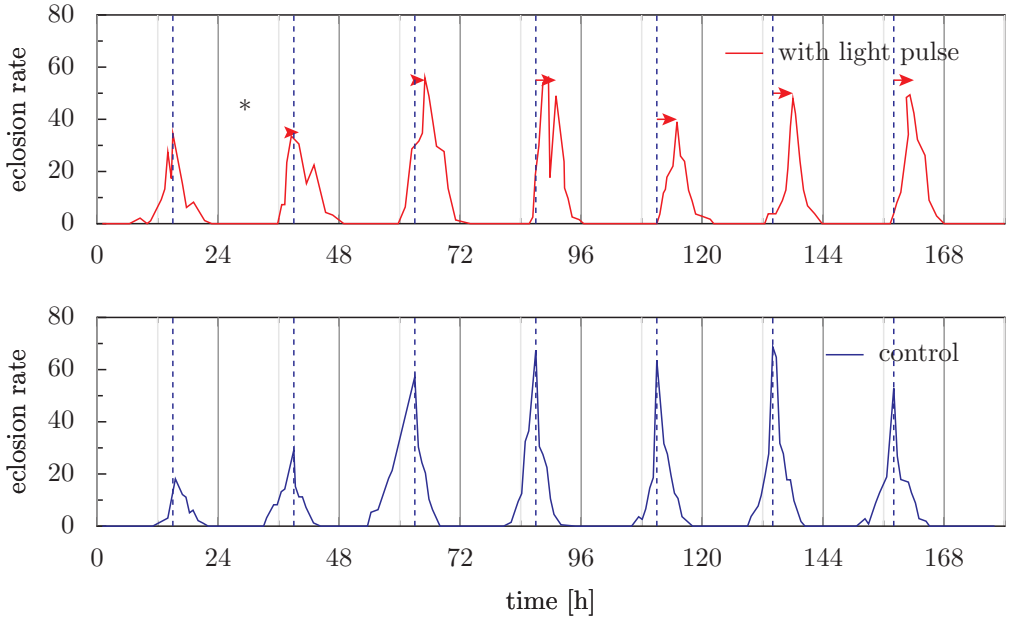

Figure 14.5: Effect of a single light pulse on the circadian eclosion rhythm of *Drosophila pseudoobscura.* Bottom curve: control without light pulse.

tain wavelength is, the less light energy is needed to obtain the effect.

Let's see how an action spectrum is obtained in the case of the eclosion rhythm. We know, that a light pulse shifts the phase of the eclosion rhythm. We have learned also, that the direction and amount of the phase shift depends on the phase in the eclosion cycle, at which the light pulse is applied. A light pulse briefly before the midnight point of the eclosion rhythm will delay the rhythm, the same pulse administered a few hours later will advance it.

We now use different wavelength of light and different energies and determine dosis-effect-curves for each color at a specific phase (figure 14.8). The higher the energy of the light, the stronger the phase shift. From the dosis-effect-curve we can determine the amount of light of a certain wavelength, which phase shifts the rhythm by, let us say, four hours. We next plot the amount of light (for instance the number of quanta) needed for this effect as a function of the wavelength. This is an action spectrum (figure 14.9). Next we compare it with absorption spectra. We might afterward conclude, that a flavoprotein is responsible for the phase shift of the eclosion rhythm by light.

An action spectrum for advances (light for instance given at phase CT20) and delays (CT17) was obtained by [453]. It showed a broad peak of sensitivity between 420 and 480 nm. Wavelengths longer than 540 nm were ineffective. The spectra for advancing and delaying the rhythm looked alike. This suggests that the same photo-pigment is involved. However, advancing the rhythm took several days of transients, whereas delay shifts were completed after one day already.

Using weaker light, advancing the rhythm

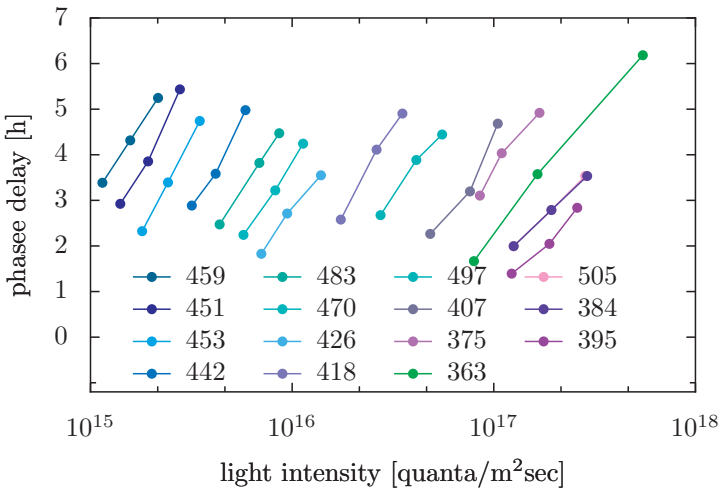

Figure 14.8: *Dosis-effect curves of phase shifting the eclosion rhythm in* Drosophila*: Different light intensities (x-axis) of various wavelengths (see the numbers at the curves, in nm) were used to irradiate batches of pupae at a certain phase of their circadian rhythm (CT 17). At that time phase delays are obtained, and higher intensities (more quanta) shift more, as shown by the slope of the curves. Certain wavelengths of light such as 459 and 451 nm are more effective than others such as 384 and 395 nm, as shown by the lower numbers of quanta needed for phase shifting. The x-axis is a logarithmic scale. These data are the basis for the action spectrum in figure 14.9. Details in [606]*

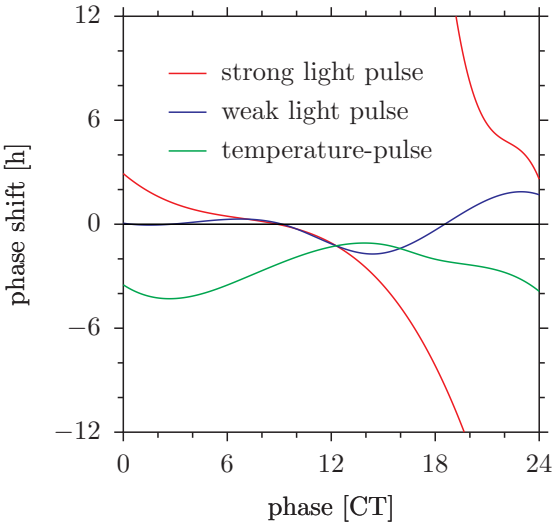

Figure 14.6: *Effects of single light- and temperature pulses on the eclosion rhythm of* Drosophila pseudoobscura. *Many experiments of the type shown in figure 14.5 have been performed in order to obtain the data of phase shifts as a function of the phase in the circadian cycle at which they were administered. Strong light pulses lead to a strong phase response curve (red curve) and weak light pulses to a weak phase response curve (blue curve). Pulses of high temperature phase shift the rhythm in a way as depicted by the green phase response curve*

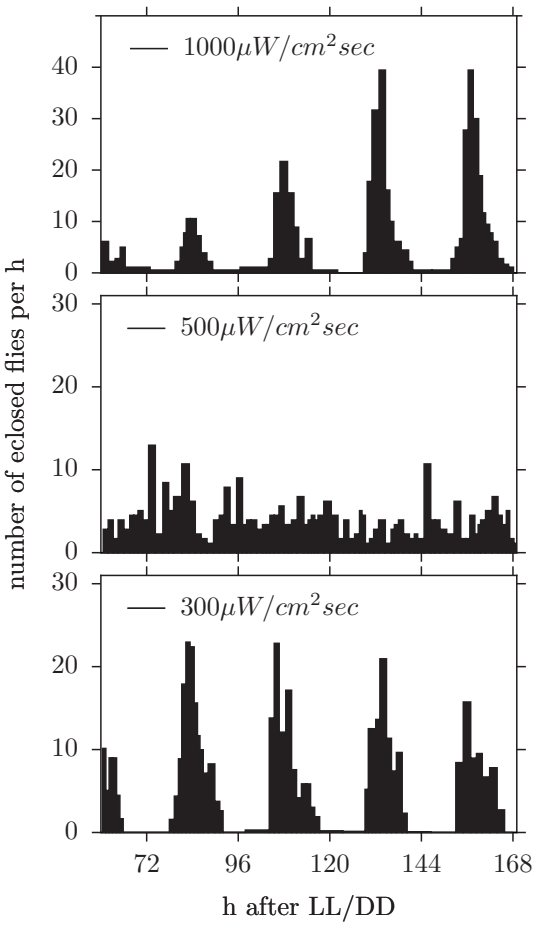

Figure 14.7: *By a special light pulse given at a certain phase the rhythmic eclosion of* Drosophila*-flies out of the puparium is prevented: The animals eclose randomly (middle). The strength of the light pulse (blue, 500 $\mu W/cm^2 sec$) has to be just between a strong and a weak reaction and must be administered at the subjective midnight (CT 18.7). 1000 $\mu W/cm^2 sec$ (top) or 300 $\mu W/cm^2 sec$ (bottom) do not lead to arrhythmicity. After [229]*

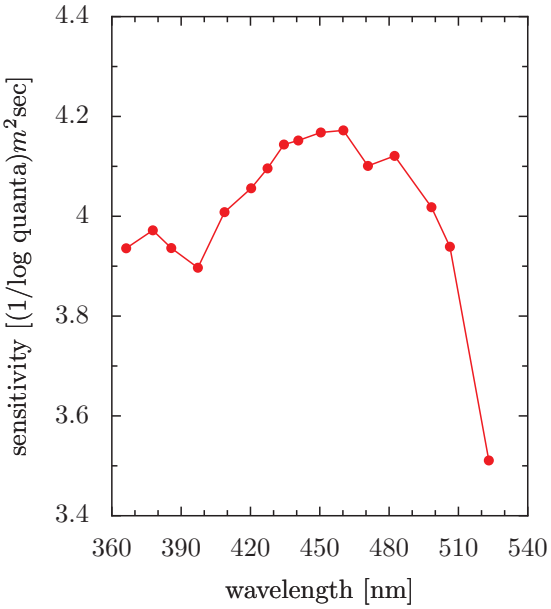

Figure 14.9: *Action spectrum of phase shifting light in the eclosion rhythm of* Drosophila*: The number of quanta needed to delay the rhythm by four hours was obtained from figure 14.8 and the reciproke plotted as a measure of sensitivity (y-axis) against wavelength (x-axis). Details in [606]*

was ten times more sensitive to blue light as compared to delaying ([228]). At higher (saturated?) light intensities these differences disappeared. These findings were interpreted as indicating two different photoreceptor pigments or different primary processes being responsible for light absorption during the advance and delay portion of the phase response curve, respectively. But an alternative explanation is more likely (see on page 329).

A more detailed action spectrum for delay phase shifts (at CT 17) of the eclosion rhythm was obtained by [790] (see figure 14.9). Blue light of 457 nm is the most effective light. Additional maxima are found at 473, 435 and 375 nm. The dose response curves for wavelengths between 400 and 500 nm were parallel lines (figure 14.8). This speaks in favor of only one pigment as the photoreceptor. The maxima of the action spectrum point to a flavoprotein and not to a carotenoid (rhodopsin) as the photoreceptor molecule for phase delays.

This conclusion is supported by results of [1611]: Flies grown on a diet lacking carotenoids showed a *visual* sensitivity of their compound eyes which was reduced by three orders of magnitude. The sensitivity of the *circadian eclosion rhythm* was however unaffected. Metamorphosing flies of mutants lacking the compound eyes and larva before pupation were still able to synchronize their eclosion rhythm ([384]). This shows that the compound eyes are not necessary for phase shifting the eclosion rhythm and that other photoreceptors are involved. These are Hofbauer-Buchner eyelets (figure 14.14) which stem from Bolwig organs, the larval photoreceptors ([606]). They project directly to the larval circadian pacemaker neurons, and their terminals completely overlap with putative dendritic trees of the latter ([741]). In mutants that lack extraretinal photoreception, functional larval

eyes are necessary to entrain the molecular rhythms in the pacemaker cells ([740]). Cryptochrome is used as the photoreceptor molecule in these organs ([740], [739]). Finally, certain neurosecretory cells, the s-LN$_v$, which are the pacemaker cells for driving circadian eclosion and contain cryptochrome, are synchronizing eclosion even in the absence of the other photoreceptors (reviewed by [604], [603], [609]). The inputs of light into the clock are thus quite well understood. However, the outputs to the target organs, tissues and behavioral centers are less well understood. How much is known about the rhythmic control of eclosion is the topic of the following subsection.

14.1.5 Physiology of eclosion and its rhythmic control

The brain of a *Drosophila* larva is shown in figure 14.10 schematically. The sLN$_v$ are the relevant pacemaker cells controlling eclosion in a circadian fashion. DNs are the primary targets of these master clock cells. The circadian signals are electrically transferred via interneurons and humoraly via neurosecretory cells in the pars intercerebralis and pars lateralis. From here the relevant centers in the thorax and elsewhere are reached which are responsible for eclosion.

The physiological and hormonal events which lead to the shedding of the old cuticle in insects ('ecdysis') are reviewed by [467] and the circadian control of it by [689]. Involved are the hormones 20hydroxy ecdyson ('20E', [1236]), the prothoracitropic hormone PTTH, the eclosion hormon (EH, [1489]), the ecdysis-triggering hormone (ETH, [1614]) and the crustacean cardioactive peptide (CCAP, [414], [472]).

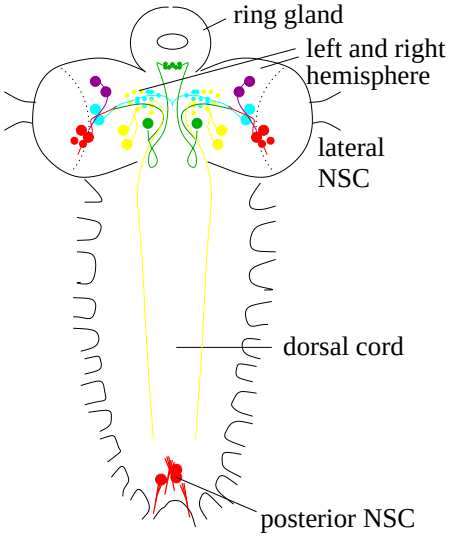

Figure 14.10: *Nervous system of a 13 hour old larva of* Drosophila *seen from above with the paired hemispheres and the ring gland (top) and the extended ventral nerve cord with neurosecretory cells at the posterior part of it. Nach* [600]

In figure 14.11 it is shown, which parts of the brain are involved in eclosion, and figure 14.12 shows the sequences of events leading to ecdysis and eclosion and their circadian control. A pair of neurosecretory cells in the pars intercerebralis and the pars lateralis of the brain release PTTH (which reduces the 20E production in the prothoracic glands) and EH (three different neuropeptides). Together with ETH from the Inka cells of the epitracheal glands in the abdomen ([1614]) PTTH and EH induce ecdysis. EH and ETH stimulate the neurosecretory CCAP cells (segmentally arranged in the subesophageal ganglion and the thoracic-abdominal nervous system) to release CCAP in a time gate. CCAP modulates muscle contractility probably by affecting motor neurons of the abdominal ganglia and results in the

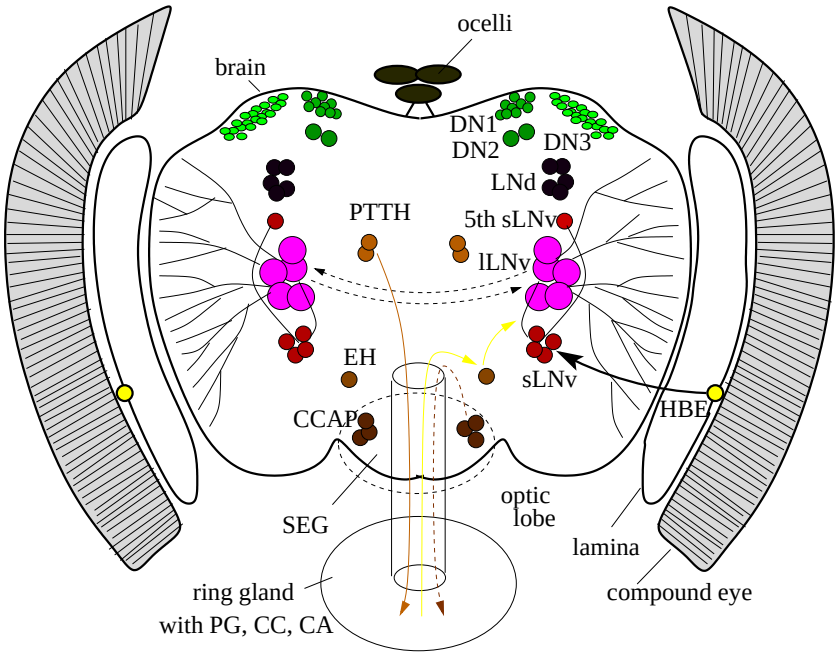

Figure 14.11: *Control of eclosion of* Drosophila *by cell groups in the brain. Decisive for the control of eclosion are PTTH and EH in the brain, CCAP in the subesophageal ganglion SEG (dashed ellipse), PG, Ca and Cc in the ring gland). The circadian system consists of the small ventral lateral neurons sLNv (red) and the large ventral lateral neurons lLNv (violet). It is connected with the dorsal lateral neurons LNd and the DN1, DN2, DN3-cell groups and additionally with the structures, which are responsible for eclosion (PTTH, EH, CCAP). Light can synchronize the eclosion rhythm via photoreceptors (compound eye, ocelli and Hofbauer-Buchner-eyelets HBA, gold and yellow). Since the figure is confusing, the important steps in the control of eclosion are shown in figure 14.12. After [1049] and Helfrich-Förster*

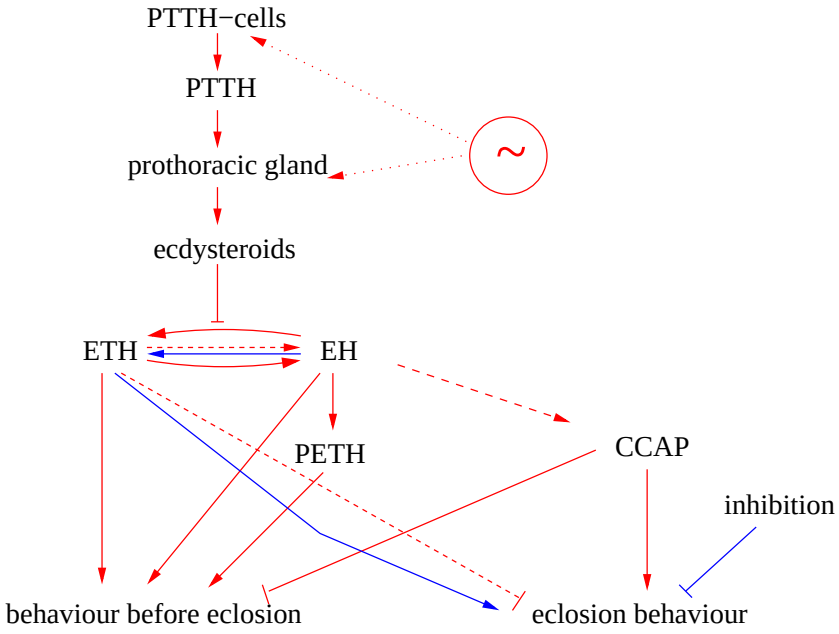

Figure 14.12: *Sequences of events leading to ecdysis and eclosion and their timing by a circadian clock (red circle with oscillation and dotted arrows, since uncertain). Two models are shown. Solid red paths are common to both. A pair of neurosecretory PTTH cells in the* pars intercerebralis *of the pupal brain release PTTH (a neuropeptide). It induces ecdysteroide release of the prothoracic gland into the hemolymph. Before a molt its concentration is, to begin with, high, and decreases strongly later on. Molting and eclosion can only occur if the concentration of the ecdysteroids in the animal is low. The eclosion-inducing hormone ETH from the Inka cells of the epitracheal glands in the abdomen and the eclosion hormone stimulate the neurosecretory CCAP cells in the subesophageal ganglion and in the thoracic-abdominal nervous system to release CCAP. It ends the pre-eclosion behavior and modulates muscle contractility and results in the final ecdysis behavior. After [1049]*

final ecdysis behavior ([471]).

The circadian system controls two events:

1. The release of PTTH from the brain and the reduction of 20E in the prothoracic glands ([1498] in *Rhodnius prolixus*)
2. The release of EH from the brain ([44], [1489]).

In *Drosophila* the PTTH cells and the EH cells are both located in the dorsal brain and the terminals of the s-LNv overlap with putative dendritic fibers from the EH cells (double labeling studies of [108]). It is likely that the PTTH cells are also innervated by the s-LNv cells ([1614]). The CCAP neurons in the subesophageal ganglion arborise dorsally and may reach the dorsal protocerebrum, thus allowing contact with the s-LNv (figures 6A,B in [1608]). Furthermore, DN2 fibers and CCAP-fibers overlap ([1140]). However, other pathways are implied, since rhythmic eclosion of fruit flies continues after ablation of the EH neurons ([981]). The PTG is a good candidate, since it shows a circadian rhythm ([368]). If per/tim expression in the PTG is overexpressed, the glands remain arrhythmic ([1049]). Thus, presumably multiple, redundant pathways mediate the circadian gating of pupal eclosion.

14.2 Locomotor activity of *Drosophila* is controlled in a circadian way

We now turn to the locomotor activity of *Drosophila* which is also controlled by a circadian clock. Activity can be measured with an infrared light beam device. In the example given in figure 14.21 the animal stayed for 3 days in a 12:12 hour light-dark cycle. Activity of the flies was restricted to the light period. The light-dark cycles had synchronized the clock which controls locomotion. Thereafter the animals were kept under weak continuous red light. Now the endogenous clock shows up: Since its period length is not exactly 24 hours (in contrast to the eclosion rhythm), each day the onset of activity is somewhat later (in this particular fly; another fly might have a faster clock and therefore each day a somewhat *earlier* onset of activity). From the slope of the line connecting the onsets of activity of sequential days the period length can be calculated. It amounts to 24.6 hours in the example given in figure 14.21.

With different intensities the period length can be influenced by continuous light[1] ([814]). This is in contrast to temperature. If temperature is varied, the period length stays (almost) constant. This temperature compensation of circadian rhythms was mentioned already before as a characteristic property of circadian clocks (see page 38) and holds also for the eclosion rhythm (see figure 14.4) and the locomotor activity rhythm of *Drosophila* ([814]).

Drosophila reared from the egg stage in continuous darkness shows in the adult stage a circadian locomotor activity rhythm. It is, however, not synchronous between the individual flies. The rhythm can therefore be induced without a light signal. Light in the embryonic stage does not synchronize the rhythm of the animals. Light in the first larval stage does ([1360]).

[1] Continuous light stronger than 10 lux induces arrhythmicity in the flies locomotor activity

14.2.1 Mutants

Mutants of *Drosophila* which differ in their circadian activity pattern played and play an essential role in elucidating the mechanism underlying the circadian clocks. The most important one are compiled in the upper part of table 14.1. Eye mutants were used to find out which of the photoreceptors are responsible for synchronizing the locomotor activity ([609], lower part of table 14.1; see also subsection 14.2.4).

14.2.2 Multioscillatory system

Out of the many events controlled in a circadian way in *Drosophila* two examples (which are also the most studied) were presented: Eclosion and locomotor activity. Both are behavioral rhythms. We are now asking for the clocks controlling them. First of all: Is there one clock responsible for the control of them or several? If these clocks are different, how are they related to each other and how do they interact? Secondly: Where are they located (see subsection 14.2.3)? Finally: How do these clocks function (subsection 14.2.5)?

In *Drosophila* peripheral tissue and different organs are controlled by autonomous circadian clocks.[2] They were found in the compound eyes ([234]), in the chemosensory cells of the antennae ([1166], [830]), in the malphigian tubules ([592], [498]) and in the prothoracic glands ([368]). However, these cells and organs are not important for eclosion and activity rhythms. Instead, eclosion and locomotor activity are controlled by circadian centers in the brain. The eclosion clock works in the pupa. In *Drosophila pseudoobscura* it takes 7 days for a larva after puparium formation to metamorphose into a fly (20^0C). The eclosion clock sets the time window for eclosion. If the fly has eclosed, a locomotion clock restricts the locomotor activity of the animal to the day time. Mind you, *Drosophila* is a day-active animal. If the animals are kept under constant conditions (as for instance weak red light), they are active during their *subjective* day. It might well be that the same clock controls both events: First the gate for eclosion, afterward the gate for activity is set.

Are eclosion and locomotor activity controlled by the same circadian clock?

The eclosion rhythm has under constant conditions a precise 24-hour-measure, and it was explained before why this is so. The locomotion clock, however, runs under constant environmental conditions usually somewhat faster or slower than 24 hours in the individual flies. This seems to speak against a single clock controlling both events. However, the clock could change its speed after eclosion. Thus, eclosion and locomotion would still be controlled by one clock. It was, however, shown that the locomotion clock runs already in the pupa, and with a speed which differs from the one of the eclosion clock ([945]).

On the other hand, mutants of *Drosophila melanogaster*, the eclosion clock of which is faster or slower as compared to the wild type, possess also a correspondingly faster or slower lo-

[2] This was demonstrated by inserting a reporter gene of the luciferase into the cells of the body. It was attached to a promoter which is controlled by the circadian clock. It turned out that the light emission of the different tissues was circadian. They could be established even in tissue cultures. Using light pulses the phase of these rhythms was shifted ([1166]).

Table 14.1: *Important mutants in circadian clock properties (upper part) and eye mutants (lower part) of* Drosophila melanogaster

mutant	name	protein in wildtype	property	remark	reference
per	period	PER	transcription regulator	negative feedback	review: [550]
tim	timeless	TIM	clock component	light resetting	review: [1359]
Clk	clock	CLK	bHLH-PAS transciption factor	heterodimer with CYC	[10]
cyc	cycle	CYC	bHLH-PAS transciption factor		[1292]
dbt	doubletime	DBT	protein kinase	degrates Per	[1178]
vri	vrille	VRI	represses clock transcription		[113]
sgg	shaggy	SGG			[963]

mutant	name	protein in wildtype	property	remark	reference
so	sine oculis				
sol	small optic lobe				
eya	eyes-absent				
oc	ocelliless				
dcry	*Drosophila* cryptochrom	DCRY			
cry^b	crybaby	CRYB	point mutation in cry gene		
norpA	no-receptor-potential-A	phospholipase C	L transduction in CE and OC		
glass					
disco	optic ganglia blind disconnected	entrain in LD, AR in DD			

comotion clock ([813]). If the mutation would concern the oscillator, this finding would speak in favor of one circadian clock which controls both events. There is, however, one mutant (*ebony*), in which the locomotion rhythm is affected (aperiodic, poor synchronization to LD cycles), but not so the eclosion rhythm ([1082]). Similar observations were made in *Drosophila pseudoobscura* ([388]). As a corollary, in the mutant *lark* the phase of eclosion was affected, but locomotor rhythms were normal in phase and period. This would speak against one clock and for a multioscillatory system.

Mutants of *Drosophila melanogaster* lacking PER, the locomotor activity of which is therefore not controlled in a circadian way, react still photoperiodically.[3] In the photoperiodic time measurement a circadian timing system is involved ([1322]). This implies, that another circadian clock controls photoperiodic timing.

Even locomotor activity might be controlled by more than one circadian oscillator. Under constant conditions optic lobe mutants exhibit often two components in the actogram, which differ from each other somewhat and drift therefore apart slowly, until they meet again after a while (figure 14.13, [597], [602]). One of these components is the morning oscillator. Even this entity seems to consist of two components. This shows up in actograms of animals the locomotor activity of which was recorded under extreme light-dark-cycles ([1237]).

Taking together these observations, we conclude: *Drosophila* possess like other insects a multioscillatory system. It controls different events at different levels in a circadian way. This system consists of autonomous clocks in cells, tissues and organs, which are synchronizable by light-dark cycles directly, but also of central clock cells in the brain which are synchronized via different photoreceptors, but also directly by light (see subsection 14.2.4). The interactions among these different components of the circadian system are not yet well understood.

14.2.3 Localization of the clocks controlling eclosion and locomotor activity

As far as the centers of the circadian control of eclosion and locomotor activity are concerned detailed information as to their localization, hierarchy, coupling and neuronal connections with inputs and outputs are studied intensively (review: localization [740], [604], light input pathways [552], [603], output pathways [602], [607], [738]). The neuroarchitecture of this system is shown in figure 14.14 together with the different photoreceptors for light synchronizing the clock cells (which are discussed in the next subsubsection 14.2.4). The pacemaker neurons controlling rhythmic eclosion and activity are located in the lateral brain and are therefore called Lateral Neurons (LN). The LN can be divided into a more dorsally located group (LNd, 5 to 8 cells) and into a more ventral one (LNv). The ventral group consists of neurons with large somata (large LNv, 4 to 6 cells) and of neurons with small somata (small LNv, 5 cells). The small LNv send projections into the dorso-frontal part of the central brain (mushrom bodies[4], calyces - centers of odor processing and

[3] the critical day-length is, however shortened by 2 hours

[4] although circadian influences of the mushrom bodies have been demonstrated, they are not necessary for the locomotor activity rhythm ([603])

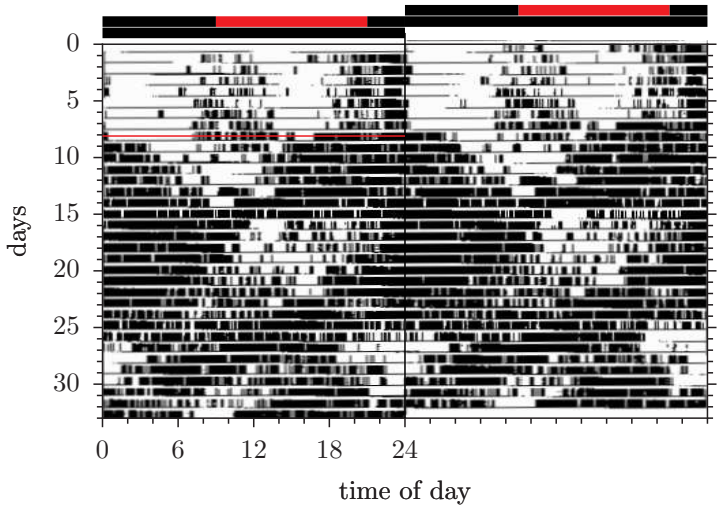

Figure 14.13: *Actogram of a* Drosophila melanogaster *fly with two circadian components seen in the locomotion pattern. In the first seven days the fly was kept in a 12:12 hour light-dark cycle. Locomotor activity consists of a morning and an evening part and the activity is synchronized to the external 24 hour rhythm. From day eight onward the fly is kept under constant darkness. A free run of the activity begins with two components: The period of the shorter component is about 22 hours, the period of the longer 25 hours. Therefore the two components drift apart from each other slowly and rejoin several times. Double plot (day 1 and 2 in first line, day 2 and 3 in second line and so on), in order to visualize the course of the two rhythms more clearly. From [608]*

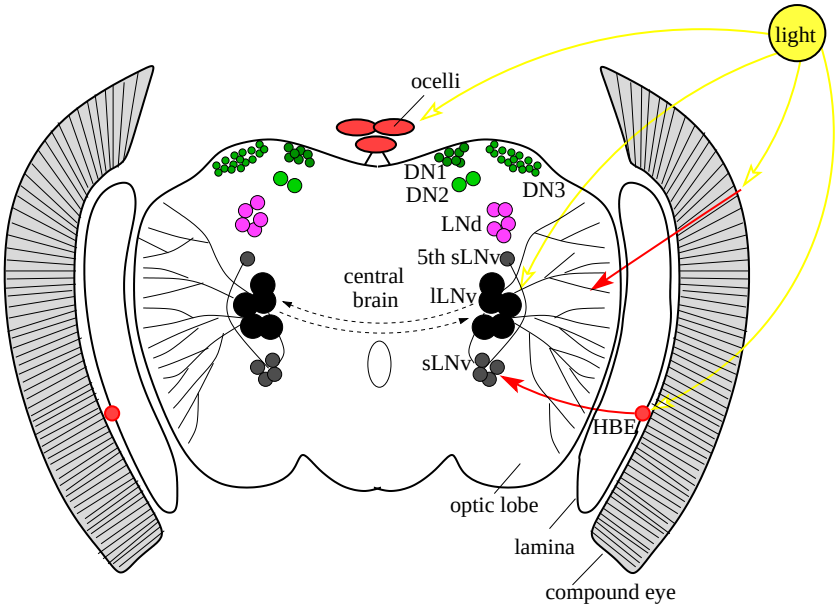

Figure 14.14: *Location of photoreceptors in the brain of* Drosophila melanogaster *for synchronizing the circadian locomotor activity rhythm. Frontal section through head with central brain, optic lobes containing lamina and medulla with lobula (not shown separately), different photoreceptors and neurosecretory cells. Light is known to synchronize the rhythm (heavy red arrows) via the compound eyes, the Hofbauer-Buchner (H-B) eyelets, and the ventral lateral neurons (sLNv, red-brown, and lLNv, lilac) and might affect the rhythm (light red arrows) via ocelli and the dorsal lateral neurons (LNd). After [608]*

learning). The LNd project to the central complex, which is a higher coordinating center of motor behaviour ([1428]) and apparently a relay station for circadian signals. The small LNv are the most important cells for circadian behavior. Three clusters of dorsal neurons (DN1, about 15 cells, two DN2 cells, and about 40 rather small DN3 cells) are found in the dorsal superior brain. In the larval brain only the small LNv, DN2 and two cells of the DN1 are found (by using antisera against the clock proteins PER and TIM).

The neural connections were elucidated by immuno-staining a neuropeptide, the pigment dispersing factor PDF, and by using a *per*- or *tim*-driven GAL4 in transgenic flies to express marker genes like lacZ or sequences encoding green fluorescent protein (GFP) or TAU protein. The *per*/*tim* expressing lateral neurons project mainly into the dorsal protocerebrum where their terminals overlap. Out of the DN clusters only the DN1 project to other brain areas such as the accessory medulla aMe and perhaps further into the optic lobe. Other DN1 cells project to the esophagus. The major projections of the DN1 run as a dorsal commissure to the contralateral side of the dorsal brain. The DN2 neurons run in the same commissure, but the DN2 somata do not contain clock proteins. The DN3 cells run more ventral and seem to terminate in the ipsilateral dorsal brain. Here they overlap with some fibers from the LNd and the DN2 terminals. The LNd axons loop along the outer surface of the lateral horn neuropil and form a dorsal branch which runs parallel to the DN3 fibers, and a ventral branch crossing the mid-line below the DN1/DN2 commissure.

The main projections of the clock cells terminate in the superior protocerebrum. This part of the brain connects to most sites of the brain and contains furthermore the neurosecretory cells. Circadian signals from the clock cells can thus be transferred via electrical or humoral pathways to effector organs. All clock cell neurons seem to be mutually connected and are thus able to signal circadian time to the dorsal protocerebrum and perhaps via the large LN also into the optic lobe. However, the various clock cells might play different roles in the circadian system.

The small LNv and LNd are the most likely clusters representing the pacemaker of the circadian behavioral rhythms. They are sufficient and essential for eclosion and locomotor activity, as discussed in detail by [602]. Even a single functional LN is sufficient, as shown by using disco mutants in which occasionally one or a few of those cells remain ([601]). However, the DN *are* involved in the circadian system. For circadian eclosion the small LNv are the relevant pacemaker cells. Only they express *per* and *tim* continuously. In the adult fly the LNd, large LNv and small LNv are involved in controlling circadian locomotor activity. These clusters use PDF as a mediator neurotransmitter for downstream neurons.

Mutants lacking PDF become arrhythmic after transfer from a 12:12h LD cycle into constant conditions. So do transgenic flies in which the large LNv and small LNv are killed by activating specifically cell death genes. However, a damped rhythm with a short period can be observed for about a week in both cases. disco mutants without both types of LNv *and* without LNd are arrhythmic already after 1 to 3 days. Thus the LNd do contribute to the activity rhythm additionaly to the LNv. The large LNv do not project into the dorsal protocerebrum and may have a special function in coupling the small LNv and LNd of the two hemispheres. They might not be relevant for rhythmic behavioral output. For details and discussion see [602].

14.2.4 Photoreceptors for the synchronization of the locomotion clock and entrainment of the molecular feed back oscillator

Circadian rhythms have, as we know by now, certain properties such as free run and temperature compensation. They can be synchronized by Zeitgeber and the light-dark change is the most important. The light is perceived by photoreceptors and transformed into signals which reach the oscillator and influence it. Informations on photoreceptors for light which synchronizes the *eclosion* clock have been presented before (see subsection 14.1.4).

How light synchronizes the *locomotor activity rhythm* of *Drosophila* is shown schematically in figure 14.14. Not just one, but several photoreceptors are involved. Even if one or several of these receptors are absent (in eye mutants), the rhythm is still entrained ([598], [597], [345], [1557]). All photoreceptors have to be absent or non-functional if flies are not entrained by light-dark cycles anymore ([609]). Details are discussed in the various papers (see the review by [603]).

Compound eyes are used in order to recognize the spaciotemporal structure of the visual world (*form vision*). But they are also used for entraining circadian rhythms (*circadian photoreception*). Although the locomotor activity of mutants which lack compound eyes (or animals which are carotenoid depleted) is still entrainable by light-dark cycles, their action spectrum is different from that of the wildtype ([110], [1102]).

The action spectrum for eyeless flies shows some similarity to the action spectrum for phase delays of the *eclosion* rhythm. The main difference lies in the sensitivity to red light. Eclosion rhythm as well as the rhythm of activity of blind mutants are insensitive to red ([600]). This suggests that rhodopsin contributes to the entrainment of the activity rhythm but not to phase shifts in eclosion. Entrainment involves tonic as well as phasic effects of light[5]. Phase shifting by short light pulses seems to be due to nonparametric effects of light. The compound eyes and the larval photoreceptors which both utilize rhodopsins as photo-pigments may mainly be responsible for the parametric effects of light. For the activity rhythm a spectral response curve[6] for phase shifts is known ([1434]). It has a maximum at 500 nm with some sensitivity to red light. Thus it resembles more the action spectrum for entrainment of activity than that of the phase shifting of eclosion. This suggests that the compound eyes do also contribute to phase shifts of the activity rhythm (nonparametric effects of light), and that differences in the composition of circadian photoreceptors in larvae and adults account for the different action spectra for eclosion and adult activity.

The circadian light sensitivity is reduced by 3 orders of magnitude in carotenoid depleted flies. This corresponds to the decrease in *visual sensitivity* of the compound eyes in carotenoid depleted flies. In the mutant *sine oculis* lacking compound eyes the circadian light sensitivity was about 2 orders of magnitude smaller as compared to wildtype flies.

[5] Tonic effects of light are also called 'parametric effects´ and deal with long lasting illuminations. Phasic effects of light are also called 'non parametric effects´ and are due to pulses of light. However, 'parametric´ and 'nonparametric´ should be restricted to the mechanism of light: Parametric effects modify the internal parameters that define the oscillating system, and nonparametric effects change the state of the system

[6] In contrast to an action spectrum spectral response curves show the responses to the same amount of differently colored light. In an action spectrum the number of quanta are determined which bring about the same response (say same phase shift)

The **ocelli** seem to play a role together with the compound eyes in the synchronization by extreme light-dark-cycles.

There are, however, other photoreceptors involved in entraining the locomotor activity rhythm[7]. One of these extraretinal photoreceptors are the **eyelets** (or Hofbauer-Buchner eyes) which contribute also to the entrainment of the adult activity rhythm ([642]).

The Hofbauer-Buchner eye is composed of 4 photoreceptor cells with numerous microvilli arranged in rhabdomeres ([1594]). The development, ultrastructure and circadian function is discussed by [605]. This eyelet corresponds to the photoreceptors in the Bollwig organ, the visual organ of the larvae. However, from the 12 photoreceptors only 4 survive metamorphosis, and the position of them is changed. The rhabdomeres exhibit rhodopsin (Rh6)-like immunoreactivity and they react with antisera against arrestin, which is required in the photo-transduction cascade ([1500]) and against the period-protein PER ([551]). As neurotransmitters histamine ([1171]) and acetylcholine ([1594]) are used. The eyes project into the brain region where the circadian pacemaker cells are located ([599], [738], [1594]), thus fulfilling the anatomical criteria for circadian photoreceptors. Analogous structures were also reported for other *Diptera*, for *Mecoptera*, *Trichoptera* and *Coleoptera* (for review: [1594]), but their physiological significance for circadian entrainment is not yet proven.

However, the Hofbauer-Buchner eyes are not the only extraretinal circadian photoreceptors since in eyeless flies the dose response curves for different wavelengths differ in their slope. Indeed, even *glass* mutants that lack all internal and external eye structures were still entrainable and phase shiftable ([600], [551]). It was shown that the circadian **pacemaker cells** (LNv) are also photo-receptive and that cryptochrome is used as the light absorbing molecule ([370], [216]).

In summary, *Drosophila* uses multiple photoreceptors for entrainment. Larvae and adults utilize the blue-light photopigment cryptochrome, which exerts its action in the pacemaker neurons. Additionally, in adults extraretinal and retinal eyes play a further role in adjusting activity to the environmental changes in light intensity.

Why are multiple photoreceptors used? It may enable circadian systems to use the different qualities of the environmental light. The signal-to noise problem is reduced by combining several inputs. Natural light-dark cycles do not simply consist of white lights-on and lights-off, but of slowly changing intensities during twilight. [180] proposed that organisms choose the very low pre-dawn and post-dusk light intensities for measuring day-length: They are reliable reference points of the seasons. During natural twilight at dusk and dawn the quality of light changes in its amount, its spectral composition and in the position of the sun relative to the horizon. Organisms could use these features depending on their ecology and strategy for sampling light (see [1249]). Entraining by dawn and dusk is more effective than lights-on /off programs in all animals tested so far including man ([434]).

Furthermore the different photoreceptors seem to play a different role for the

[7] Some indications were: [110] found two maxima in the action spectra for entrainment of activity of the wildtype and of eyeless flies. Furthermore, in dose response curves the lines representing the percentage of synchronized flies as a function of light intensity were not parallel for several of the tested wavelengths. Interestingly, these dose response curves were also not parallel in eyeless flies suggesting that even eyeless flies utilize different photoreceptors for entrainment.

synchronization of the circadian rhythm and for direct light effects ([370], [609], [1237]). The compound eyes are responsible for a direct light effect in the activity pattern of the adults and synchronize the circadian rhythm especially during extreme long- and short-days. The ocelli seem to show this effect too. They possibly affect the rhythm, which is responsible for the morning peak 2 (see page 322), whereas the compound eyes synchronize the rhythm which is responsible for the morning peak 1 ([1237]). Cryptochrome affects as a cellular photoreceptor molecule circadian rhythms, but is not mandatory for synchronizing the activity rhythm. The importance of the Hofbauer-Buchner-eyelets for the synchronization of the activity rhythms and for a direct light effect is not yet known, since so far no mutants are known, which lack these photoreceptors or in which they are defect. Experiments with cell death genes, which switch off these receptors specifically, are planned and could help to answer this question.

14.2.5 Molecular basis of circadian rhythms of *Drosophila*

What mechanisms underly the circadian clocks in the brain which control eclosion and locomotor activity of *Drosophila*? This has been studied in the last decades intensively and with much success. However, there are still many unsettled questions. Here only the essential mechanism will be described. For details and discussion of the problems see recent reviews by [1410] and [9].

Both rhythms - activity and eclosion - are controlled by a molecular feedback loop. It is generated by interactions of several *clock genes*, their products and by the degradation of these products. These genes are the period (*per*) ([813]), timeless (*tim*) ([1332]), Clock (*Clk*) ([10]), cycle (*cyc*) ([1292], doubletime (*dbt*) ([1178]), vrille (*vri*) ([113]), shaggy (*sgg*) ([963]) and *Pdp1* ([277]). The corresponding proteins are PER, TIM, CLK, CYC, DBT, VRI, SGG and PDP1 (see table 14.1). Mutations in each of these genes affect both rhythms strongly by rendering the flies aperiodic or changing the period of the free running rhythm. They are the *players* in the circadian game.

The *game* consists at the molecular level of two transcriptional feedback loops and of posttranscriptional regulations[8]. The circadian cycle starts in the late morning (figure 14.15, upper part): Transcription of the *per* and *tim* genes is activated by CLK and CYC, which are basic helix-loop helix-PAS transcription factors ([10], [1292]). They form heterodimers and bind to an E-box element (special sequences) in the promoters of the *per* and *tim* genes ([289], [1292], [558]). The *per* and *tim* mRNA levels increase throughout the remaining day. Peak levels are reached at the early evening ([1410]). The products PER and TIM reach their maximum not before late evening ([1606], [355]). The delay seems to be due to post-transciptional regulation of the *per* mRNA ([232]) and PER ([307], [1411]). One of the regulatory mechanisms destabilizes PER by DBT-dependent phosphorylation ([793], [1178]). PER is, however, stabilized by dimerisation with TIM. If a critical level of the PER-TIM dimer is reached, it enters the nucleus and represses its own transcription by inactivating the CLK-CYC transcriptional activator (the DNA binding ability is lost, [289]).

[8] such as heterodimerization, nuclear import, phosphorylation of clock proteins by kinases, targeting for degradation by ubiquitin proteasomes

Due to the lag between mRNAs and proteins, this negative feedback results in a stable cycling in *per* and *tim* mRNA and protein levels.

There seem to be two connected negative feedback loops: the *per*/*tim* loop, in which transcription is activated by CLK-CYC and repressed by PER-TIM, and a Clk loop, in which transcription by CLK-CYC is supressed and by PER-TIM derepressed ([501]).

The two loops allow clock controlled genes (ccg's) to induce in different physiological processes different phases of their rhythms. Loop one would have a maximum at the end of the night, loop two at the end of the day.

Not all components seem to be discovered yet. Recently, [82] reported that the cAMP response element binding protein (CREB) also participates in the feedback loop. It promotes oscillations of PER and TIM. More such clock factors might be found.

14.2.6 Molecular basis of synchronization

To understand circadian photoreception in the LNv we needed to know how circadian rhythms are generated at the molecular level in these and other cells of *Drosophila*. If the feedback loop just described is the basis of the overt rhythm, it must be entrained by light-dark cycles, phase shifted by light pulses and attenuated by continuous light. This is achieved through light dependent degradation of TIM; other clock proteins are not degraded by light ([664], [865]). Under continuous light the TIM level is permanently very low. Because PER must dimerize with TIM to be protected from degradation, the PER level is low, too ([1179]). *per* and *tim* mRNA remain at a median level similar to those of the per^0 and tim^0 mutants ([1189]). Consequently the clock genes and proteins do not oscillate under LL. This would explain the arrhythmicity found in continuous light.

If TIM degradation is measured in the LN after short light pulses it correlates well with the amount of phase shifts in the activity rhythm ([1598]). The spectral response curves for TIM degradation and for behavioral phase shifts show maximal responses to light of 400 to 450 nm. Thus both events seem to be causally related. TIM is degraded also in the absence of PER and thus in the absence of a functioning circadian clock ([1434]). It does furthermore not depend on functional compound eyes ([1593]). This indicates that circadian resetting is mediated via TIM through an extra-retinal pathway, as behavioral studies had shown already.

All these results indicate that TIM degradation is crucial in circadian light perception.

TIM degradation does not always occur after lights-on. For instance, in the larval stages TIM cycles in anti-phase to the LN in two cells of the so called Dorsal Neurons (DN) (high during the day, low during the night, [741]). The biological significance is unclear. But it shows that TIM can be regulated differentially and that it is not light-sensitive by itself. Somehow the light signal must be transduced biochemically through blue-light absorbing photo-pigments to TIM.

The blue light absorbing photo-pigment *Drosophila*-cryptochrome (CRY) is involved in transducing light to TIM ([369], [1412]). Cryptochromes are flavoproteins (review: [209]). The cryptochrome absorption spectrum corresponds to the behavioral action spectra of *Drosophila* ([1363]).

The reasons given in petite indicate that the Cry gene transcription is clock-controlled, but the CRY protein level is controlled by light and independent of the clock molecules.

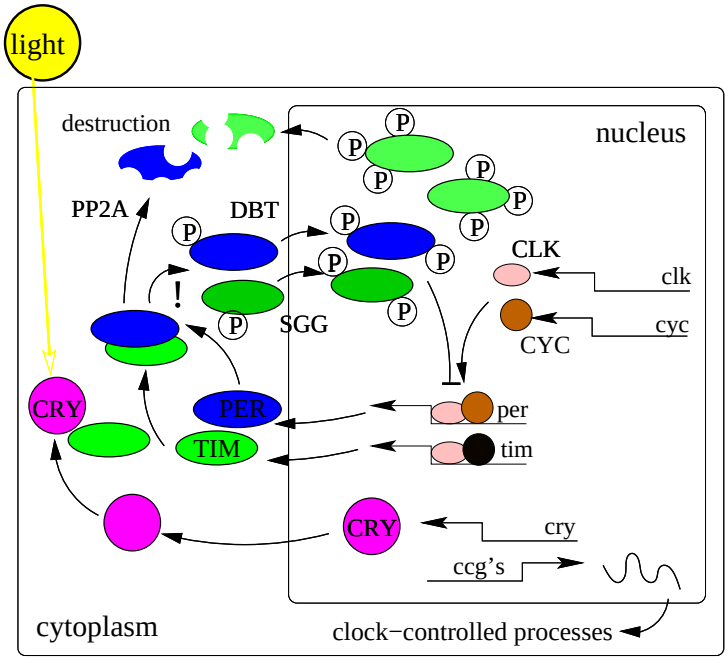

Figure 14.15: *Molecular model of the* Drosophila *clock: The clock-genes* per *and* tim *express PER (blau) and TIM (green) clock proteins. They are phosphorylated in the cytoplasm (lighter colors) and form a dimer. This is able to pass the nuclear membrane. By further phosphorylation it inhibits (grey arrows) the transciption factors dCLK and CYC (reddish) and transcription of PER and TIM is stopped. The existing PER and TIM decreases, because the phosphorylated proteins are degraded (grey). Light (red, arrow, top) is perceived by photoreceptors and changes in so far unknown steps the* Drosophila *cryptochrome DCRY. This leads to the degradation of the PER/TIM dimer. After [568] and [278]*

Changes in the Cry gene dose correlate with changes in light resetting of the activity rhythm. A lower Cry gene dose reduces the magnitude of phase shifts ([364]). Overexpression of Cry leads to larger phase shifts ([369]). Both occurs at lower light intensities only. This indicates that the system is saturated when CRY exceeds a certain level. The over-expression is less striking and more variable in the advance zone of the PRC (at ZT21) than in the delay zone (at ZT15). The reason for this might be, that at ZT15 the CRY level is still rather low and could probably be raised considerably by over-expression. Thus the effects of light on CRY can be amplified; at ZT21, CRY is already close to its peak level and possibly saturated. Therefore only small or no effects on behavioral responses are expected after CRY over-expression ([369]). This implies that the circadian system of normal flies should respond less well to weak light in the delay zone than it does in the advance zone due to the different levels of available CRY. This was found before ([228]) but interpreted wrongly.

DCRY plays thus an important role in circadian photoreception. Apparently TIM is a direct target of DCRY. It was indeed shown recently that DCRY changes its photochemical conformation after illumination: DCRY is now able to move into the nucleus and to interact with the PER/TIM complex ([216]). This interaction inactivates the PER/TIM complex and it does not participate anymore in the negative feedback loop. Degradation of TIM is not the first step in circadian phototransduction, but a consequence of DCRY blocking PER/TIM. This might explain the hypersensitivity to light of the per^s mutant ([812], [1320], [551], [1434]).

DCry is expressed in the LNv, the pacemaker neurons ([364]). The same appears to apply for *Drosophila* larvae. The small LNv are present from the first larval instar onward and show a cycling in the level of PER and TIM ([741]). The larval small LNv are apparently entrained by cryptochrome *and* the larval eyes ([1412], [606]). They do not entrain when both pathways are impaired. It has to be shown whether the same is true for entrainment of the eclosion rhythm.

14.2.7 Molecular basis of temperature compensation

Temperature compensation of the cycling length in circadian rhythms has been mentioned several times before (see for instance the examples in figures 4.6, 6.17) and different models have been proposed (see for instance figure 5.6 or section 16.4, reviewed by [1285]).

In *Drosophila*, clock mutants with aberrant temperature compensation are known. For instance, increasing temperature lengthens the period of behavioral rhythms in per^L ([814], [1271]). Since a normal chemical reaction is speeded up by increased temperature and many ultradian rhythms do so too, this 'overcompensation´ seems to be special. One could argue, that this mutant has a lower level of PER, and it is known that period increases with decreasing amounts of PER. Thus, the defect in temperature compensation could be due to the changed PER level and not to an aberrant temperature compensation. However, in other *per* mutants such as per^s the clock runs faster at higher temperatures. A defect in the temperature compensation mechanism is therefore more likely. The molecular mechanism is not yet understood (see discussion in [553]).

A region in the middle of PER might be important for temperature compensation. A PAS/C domain association could for instance compete with a PAS/PAS association. The en-

hancement of PAS/PAS by higher temperature might be blocked by effects of the PAS/C domain resulting in temperature insensitivity of the period length ([533]). Another region of the PER protein upstream of the TG repeat, but downstream of the C-domain might be responsible for temperature compensation ([1146]). However, other parts of the clock mechanism such as *tim*, might be involved.

Temperature compensation in *Drosophila* has also been dealt with by using models ([876]). [1281] extended the Goodwin oscillator (see figure 16.15) by introducing a monomeric and an oligomeric form of PER protein. The equilibrium between the two forms depends on temperature. In the per^L mutant increasing temperature shifts the equilibrium to the monomeric form and both forms are more slowly degraded as in the per^+. In the per^S mutant the equilibrium is undisturbed, but PER^S is degraded faster ([1282], see figure 16.11 and 14.16). In the model, the influence of temperature is realized via clock protein *turnover* control.

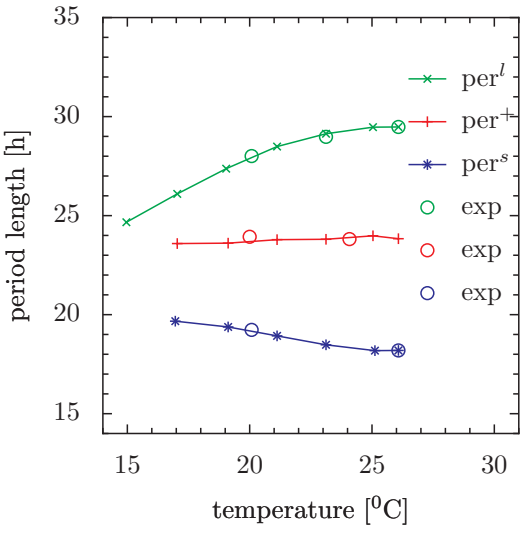

Figure 14.16: *Temperature compensation of* Drosophila *locomotor activity rhythm by using the Goodwin oscillator (figure 16.15) model with negative feedback. Temperature dependencies of* Drosophila *mutants per^L (green) and per^S (blue) are very well predicted (circles are predictions, triangles experimentally found) and temperature compensation of wild type respectively per^+ holds. After [1282]*

14.3 Rhythms and life duration

Life duration of *Drosophila melanogaster*-flies is shortened if the animals are kept in days shorter or longer than the normal 24-hour-day ([1164], figure 14.17).

In other flies similar results were obtained: [34] kept *Phormia terrae novae* - flies in a 12:12 hour light-dark cycle. The light-dark cycle of one of the groups was delayed each week by 6 hours (as if the animals were flown to the United States). In another group the light-dark cycle was each week advanced by six hours (as if the animals were flown to the east). A third group was 'flown' in one week westward, in the next week eastward, there-

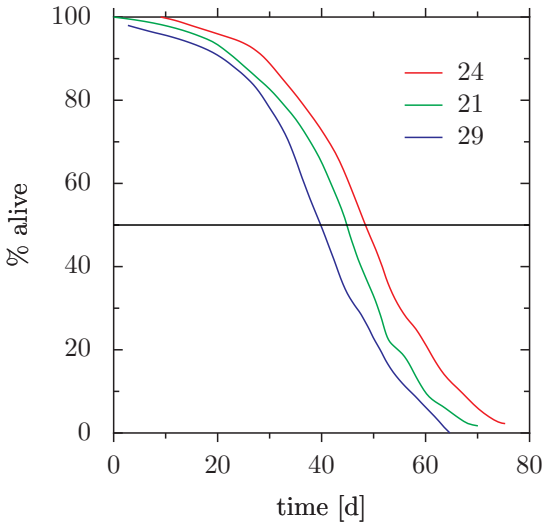

Figure 14.17: *Life duration of* Drosophila melanogaster *under non-24-hour-cycles. At time 0 (x-axis) all flies (100%) of the groups which were kept in a 24-hour, 21-hour and 27-hour light-dark-cycles were alive. With time more and more flies died, and the death rate in the non-24-hour-cycles was larger. After [1164]*

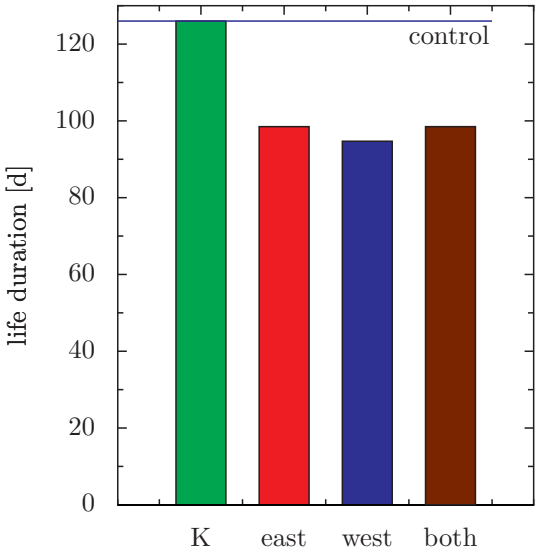

Figure 14.18: *Life duration of* Phormia terrae novae *- flies after shifts of the light-dark cycles by 6 hours to earlier (east) or later times (west) or alternating after earlier and later times (both). In all cases the life duration of the animals in the experimental groups was shorter as compared to the control group ([34])*

after again westward. In comparison to the animals which 'stayed home' life duration of these animals was shortened (figure 14.18).

In another experiment *Musca domestica*-flies were kept after eclosion in weak red light and the locomotor activity measured. They showed a circadian rhythm for some time, but most of the animals became arrhythmic occasionally. The animals with the longest life happened to be still rhythmic (figure 14.19). This indicates, that a stable circadian rhythm in older flies might be of some significance for their longevity.

We have also tested whether the life duration of *Drosophila*-flies is shortened if their circadian rhythm is stopped by a

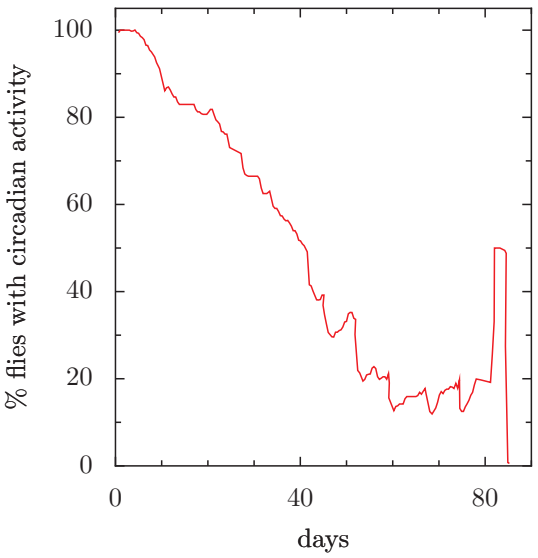

Figure 14.19: *Percentage of flies which expressed a circadian rhythm under constant temperature in weak red light. The percentage of rhythmic flies decreases with time, but shortly before all flies had died it increases considerably. That is, rhythmic flies are the one which have the longest life (Engelmann, unpublished)*

special treatment. This treatment is explained in the special story 'arrhythmia'. The results showed that there was no difference in life duration ([945]). There are, however, a number of objections against this interpretation. The arrhythmia was induced in the pupal stage. Instead of eclosing in a circadian rhythm the animals eclosed now randomly distributed. It might, however, be that after eclosion the clock is restarted again by the eclosion event itself or by other events. It might also be possible that the eclosion clock became arrhythmic, but the clock controlling locomotion not.

Further experiments should be made to clarify this interesting question, whether life duration is shortened by preventing the circadian rhythm from occurring.

14.4 How is eclosion and locomotion of flies recorded?

The results which have been mentioned so far are based on experiments, in which the rhythms of the animals had to be recorded. How this was done in eclosion and in locomotor activity will be shown in the next subsections.

14.4.1 Recording the eclosion rhythm

The rhythmic eclosion of *Drosophila* (and other insects) out of the puparium can be recorded on a population of pupae only. In earlier studies special 'Bang boxes' were used ([380]). The pupae were glued to a plate. They were hourly lifted up and released mechanically. As a result the emerged flies fell through a flat funnel into water filled vials. The vials were replaced

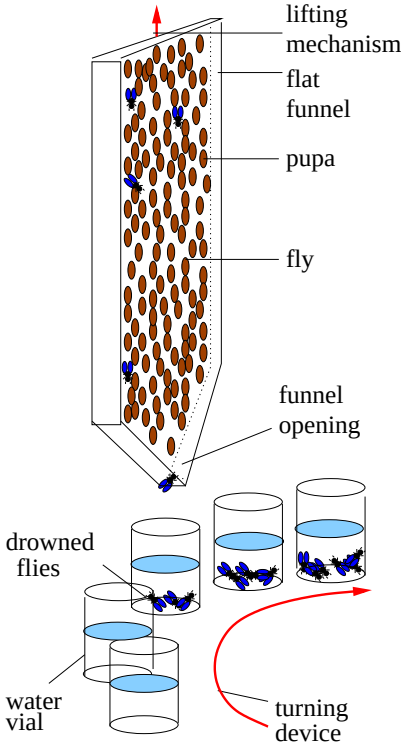

Figure 14.20: *Recording of eclosion rhythm of* Drosophila-*flies out of the puparium using a teflonized funnel and infrared-light beams at the end of the funnel*

by new ones every hour. A turning mechanism served for the replacement of the vials. The number of flies drowned in the water was counted onces per day for the hourly collections.

Later this complicated and error prone method was replaced by light beams at the end of the funnels. This method works, however, only in the dark or under red light and the funnel walls have to be slippery (using teflon or teflonized glas funnels). In light the emerged flies do not fall down immediately after eclosion (figure 14.20).

In another method the pupae are placed individually in holes of a plate. The lower side of the plate is covered with a translucent net, preventing pupae from falling through. A sooted glass plate lies on top of the pupal holder. During eclosion the flies try to escape and in doing so scrape off the soot. They die quickly and dry up. Red light is able to pass the holes in which eclosed flies have removed the soot. It falls on a photoelectric cell. The voltage of the cells is a measure of the number of flies eclosed (http://w210.ub.uni-tuebingen.de/volltexte/2009/3790/). Instead of a photoelectric cell a video camera can be mounted on top of the plates and the number of flies eclosed can be determined via an imaging analysis program.

14.4.2 Recording the locomotion rhythm

To record the locomotor activity of *Drosophila*, flies are kept individually in small vials such as spectral photometer cuvetts. A small piece of sugar and a whick of a water bottle keep the animals alive for several weeks. The locomotion is recorded with an infrared light beam ([380]). If the fly passes the light beam, an electrical signal is generated and stored. Actograms can be constructed from these data (figure 14.21). Often the informations are reduced. For instance, it is not determined, how often an animal has interrupted the light beam in a certain time window. Instead, it is determined whether a fly was active at least onces (or n times) in a certain time interval (for instance in 4 minutes). The histogram is in this case converted to a stroke actogram (figure 14.21).

Imaging analysis methods are quite versatile. With a video camera the behavior of insects can be recorded and the images analyzed using imaging programs. Much more informations can be obtained

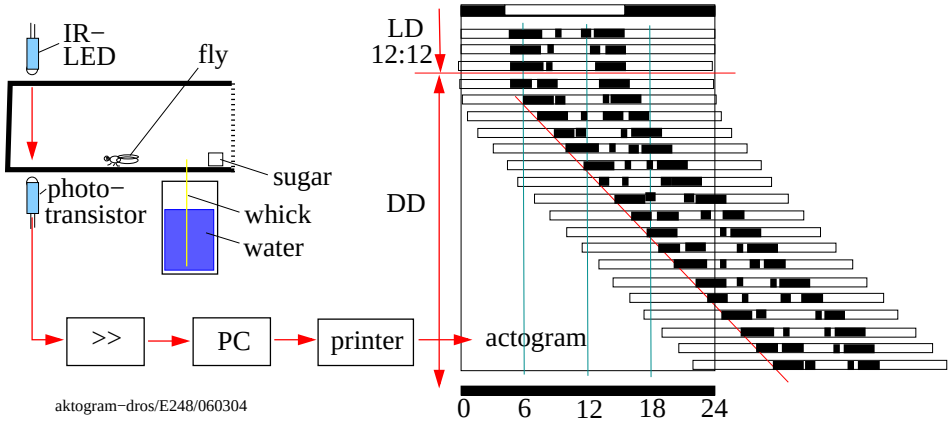

Figure 14.21: *Left: A* Drosophila *fly is confined to a small cage (a spectrophotometer cuvette) by covering the opening (at the right) with a translucent plastic lid. A wick supplies water from a container underneath, and a piece of sugar serves as food. Interruptions of the light beam consisting of an infrared light emitting diode (IR-LED) on top and a phototransistor at the bottom of the cuvette are amplified (>>) and stored and analyzed with the help of a PC. Right: An actogram of the locomotor activity of an individual fly kept in a light-dark cycle (LD 12:12) for the first 3 days and in constant weak red light (safe light and thus physiological darkness DD for the fly) afterward. From the slope of a line connecting activity onset the period length can be determined and amounts in this particular fly to 24.6 hours*

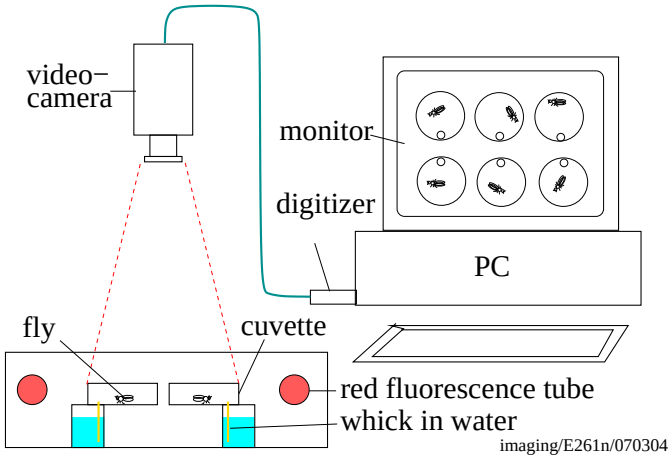

Figure 14.22: *The behavior of individual* Drosophila*-flies can be recorded with a video-camera and analyzed with a PC. Each dish contains an individual fly, a small piece of sugar (as food), and a whick to a water vial (for drinking). Furthermore the dishes are aerated through a hole with a net. Light of red luminescence tubes (which are additionally wrapped with a red foil) illuminates the animals from the side, so that they can be seen by the video-camera. The images are digitized in regular periods and converted with a program. In this way the position of the animals can be followed and the behavior (drinking, feeding, cleaning) determined. After Schuster 1988 and Engelmann, unpublished*

as compared to the procedure using light beams. Thus, the location of the animal in the cage can be determined and whether it is feeding or drinking (figure 14.22).

15 Eye clocks of marine snails

In certain marine snails (such as Aplysia *and* Bulla*) the cellular mechanisms of circadian rhythms can be studied with advantage. Their eyes contain among others neurons with circadian clocks. They control the firing of neurons. This leads to a rhythmic action potential in the eye nerve, which can easily be recorded. The processes during rest and activity in nerves is well studied. Using electrical and pharmacological treatments these processes can be influenced in defined ways.*

15.1 Introduction

Certain marine snails are well suited to study the cellular mechanisms of circadian rhythms. *Especially Aplysia* and *Bulla* have been used. Their eyes serve mainly to receive light. But they contain also neurons with circadian clocks, which control the firing of neurons. This leads to a rhythmic action potential in the eye nerve, the 'CAP' (compound action potentials, [684]).

The eyes can be isolated and kept in a proper medium for longer periods of time. It is easy to record the CAP. Processes during rest and activity of nerves are well studied. Using electrical and pharmacological treatments these processes can be influenced in defined ways[1]. The cellular mechanisms of these circadian rhythms can thus be studied with advantage.

Review articles are from [119], [118], [117], [245], [626], [688], [823], [894], [1559].

[1] Since both eyes react very similar, one of it can be used as a good control

Besides *Aplysia californica* and *Bulla gouldiana* other marine snails such as *Navanax inermis*, *Haemonea vesicula* and *Bursatella leachii plei* were studied. The systematic, occurrance and biology will be treated briefly in the following.

15.2 Systematic, occurrance and biology of marine snails

The stem of mollusks contains about 128 000 species. The sea hare *Aplysia* (order *Tectibranchia*, suborder *Anaspida*) belongs like the other marine snails to the class of Gastropods (snails) and there to the subclass of *Opisthobranchia* (figure 15.1). *Bulla* belongs to the subclass of *Cephalaspidea* in the order of *Bullidae*. *Bursatella*, *Haemonea*, *Navanax* belong likewise to the *Cephalaspidea*. *Aplysia* is day active. It occurs in the coastal intertidal zones of the Pacific ocean between central California and New Mexiko. The animals possess a reduced shell only and a large mantle (figure 15.2). The mantle is used for swimming with repulsion. The behavior of *Aplysia* is well studied. Especially the memory performance of the animal was studied by several laboratories. *Aplysia* contains a pair of lense eyes and further light sensors on the surface of the body. It feeds on algae, such as the red alga *Gracilaria*. In the laboratory salad is used as food (personal information Stefan Michel). They reproduce during the summer until the middle of the

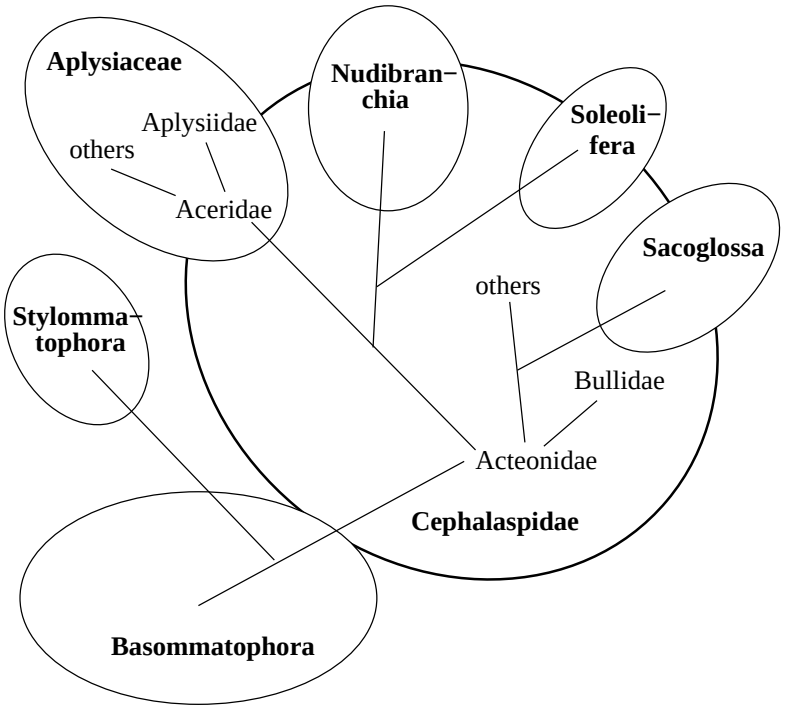

Figure 15.1: *Systematics of* Euthyneura *(before:* Opisthobranchia *and lung snails).* Aplysia *belongs to the* Aplysiidae *in the family* Aplysiaceae*. This family derives from the* Cephalaspidea*, as do other families of the* Opisthobranchia *and lung snails.* Bulla *belongs to the* Bullidae*, which are a part of the family* Cephalaspidea*. From [772]*

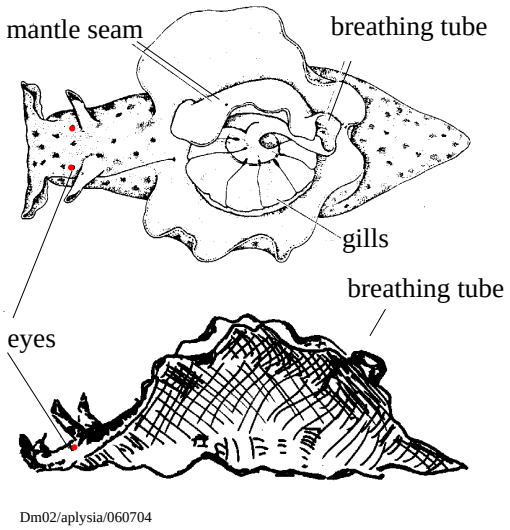

Figure 15.2: Aplysia californica *viewed from the top (top, head left with antennae, eyes (red), breathing tube and mantle, mantle seam, gill). Bottom: Animal from the side. After [885]*

Figure 15.3: Bulla gouldiana *(cloudy bubble snail) with head left, eyes in the white spots, mantle around the dark shell. Picture taken by C. Ehnert in the laboratory of S. Michel*

fall. Warm temperature (20^0C) seems to be the most important trigger to reproduce, whereas photoperiodic signals are less important (shortday increases oviposition, see [1531]).

Bulla gouldiana (cloudy bubble snail) lives in warmer oceans on sandy soil. It is an omnivore and lives on waste of the sea (detritus-feeder). *Bulla* is in contrast to *Aplysia* night active (figure 15.3).

15.3 Morphology and anatomy of the eyes and the brain ganglia

The paired eyes of *Apysia* are small ($600\mu m$) and inconspicious. The eye is a closed capsule with a central lens (figure 15.4). The eyebulb consists of two layers: A complex retina consisting of 5000 cells and a neuropil with secondary neurons (R-, D- and H-cells). The retina contains five types of photoreceptors and two types of neurons. Dorsal and ventral part of the eye differ. The neurons responsible for the CAP rhythm are found in the ventral part ([623]).

The brain of *Aplysia* consists of several head ganglia (cerebral-, pleural- and pedal ganglia). It is shown for *Aplysia* in figure 15.5 ([1110]).

The diameter of the eye of *Bulla* has a size of $500\mu m$ (figure 15.6 from [1239]). It contains about 1000 large photoreceptors, small photoreceptors, numerous pigmented support cells and about 130 neurons (Basic Retinular Neurons, BRN). They present the circadian oscillator cells and their axons run together with 2000 others through the optic nerve to the neuropil. In the optical nerve are also efferent axons from the brain. The retinal cells are coupled with each other electrically (see figure 15.7).

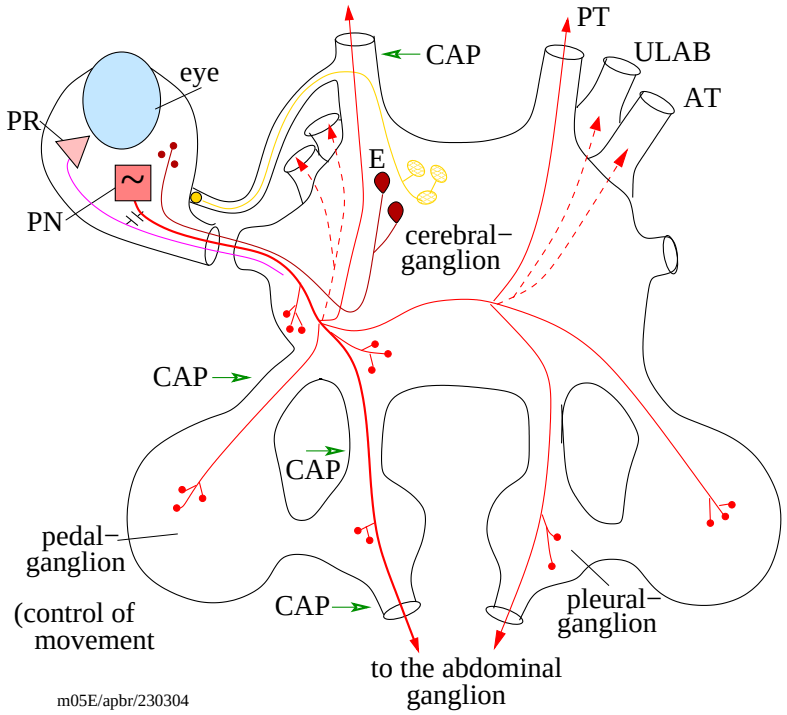

Figure 15.5: *Brain of* Aplysia californica. *The eye is connected with the brain via the optic nerve, which consists of axons from photoreceptor cells (PR, magenta), axons of the circadian pacemaker cells (PN, red, with PR fibers electrically coupled, black capacity-sign) and efferent neurons (brown) from the cerebral ganglion to the eye (E). Additional efferent neurons (yellow) run to the eye via the accessory optic nerve. The brain consists of different ganglia (cerebral, pedal, pleural and more distal onces which are not shown) and is connected with the abdominal ganglion. Thick lines (red) show projections of the pacemaker cells PN to different parts of the brain and its ganglia (shown by radioactive labeling). Short green arrows indicate regions in which compound action potentials (CAPs) of circadian pacemaker-neurons in the retina can be recorded. After [1110]*

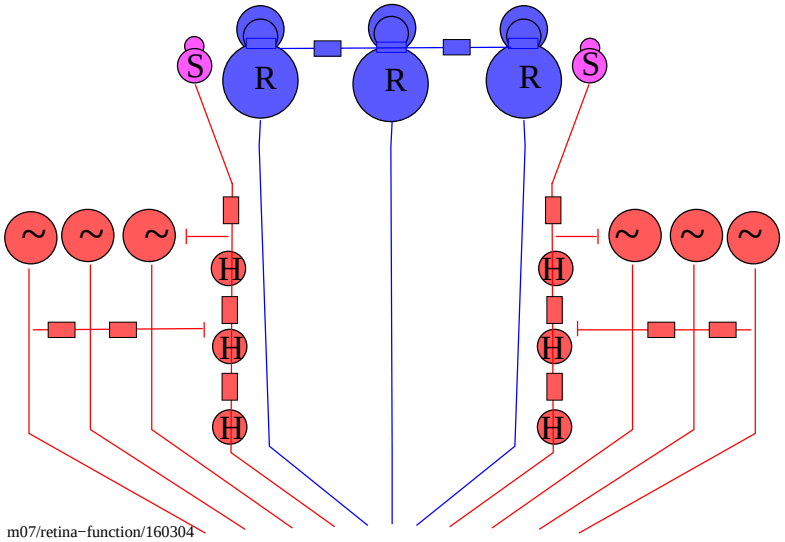

Figure 15.7: *Model of functional organization of the retina of* Bulla gouldiana. *R (blue): Large photoreceptor cells, which surround the lens of the eye, are coupled electrically with each other (blue connections with rectangles). S (violet): Small firing photoreceptor cells between the large one. H (brownish): Chain of small retinal cells, coupled electrically with the S-cells, which are temporarily inhibited during illumination. BRN (red): Basal retinal neurons, which are pacemaker cells; they depolarize during illumination and induce action potentials. Neurons of the BRN´s couple electrically with each other (brownish lines with rectangles) and inhibit (brown lines with small vertical strokes) the H cells (and S-cells?). Cells of the photoreceptor-layer seem to inhibit the BRN´s (brownish lines with rectangles). Axons at the base lead via the optic nerve to the brain. After [119]*

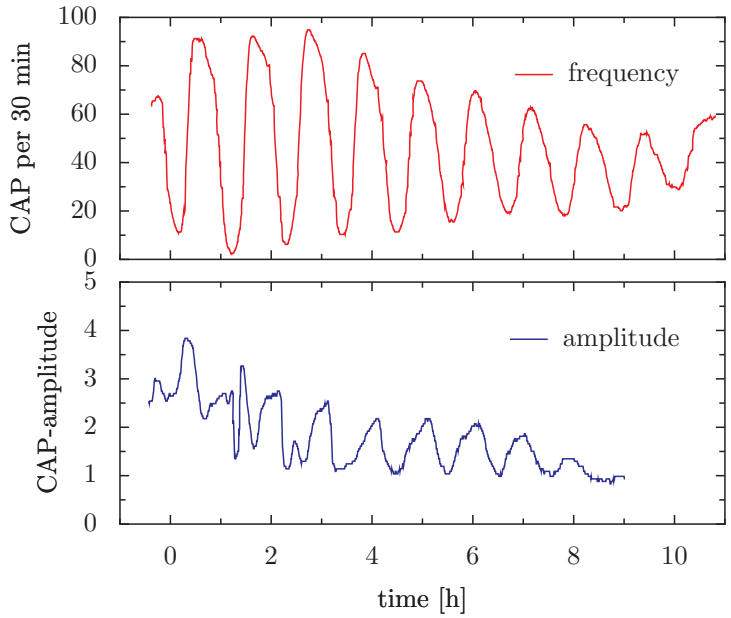

Figure 15.9: *Circadian rhythm of CAP amplitude (top, blue curve) and frequency (bottom, red curve) of isolated eyes in the dark. After [84]*

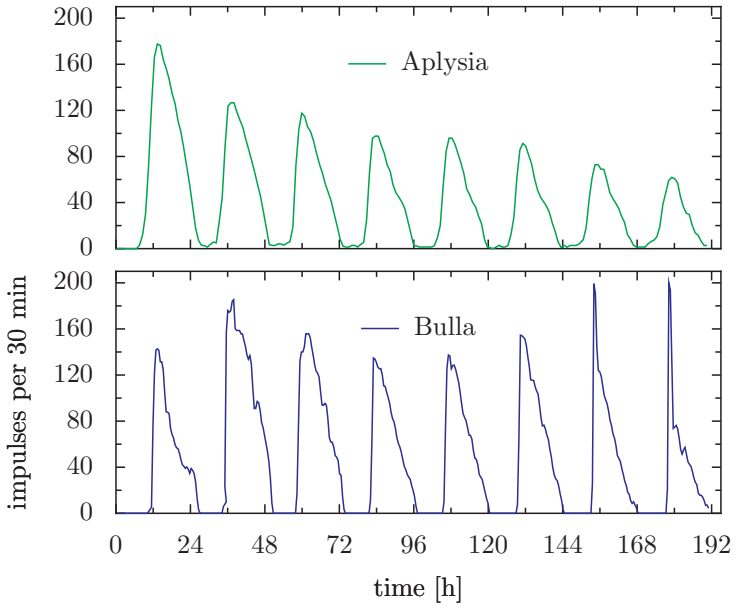

Figure 15.10: *CAP-rhythm of the optic nerves of isolated eyes of* Aplysia *(top, green curve) and* Bulla *(bottom, blue curve), recorded for eight days at 15°C after transfer from a light-dark cycle into continuous darkness.* Bulla *is night active, whereas* Aplysia *is day active. In spite of it both curves are in phase with each other. After [119]*

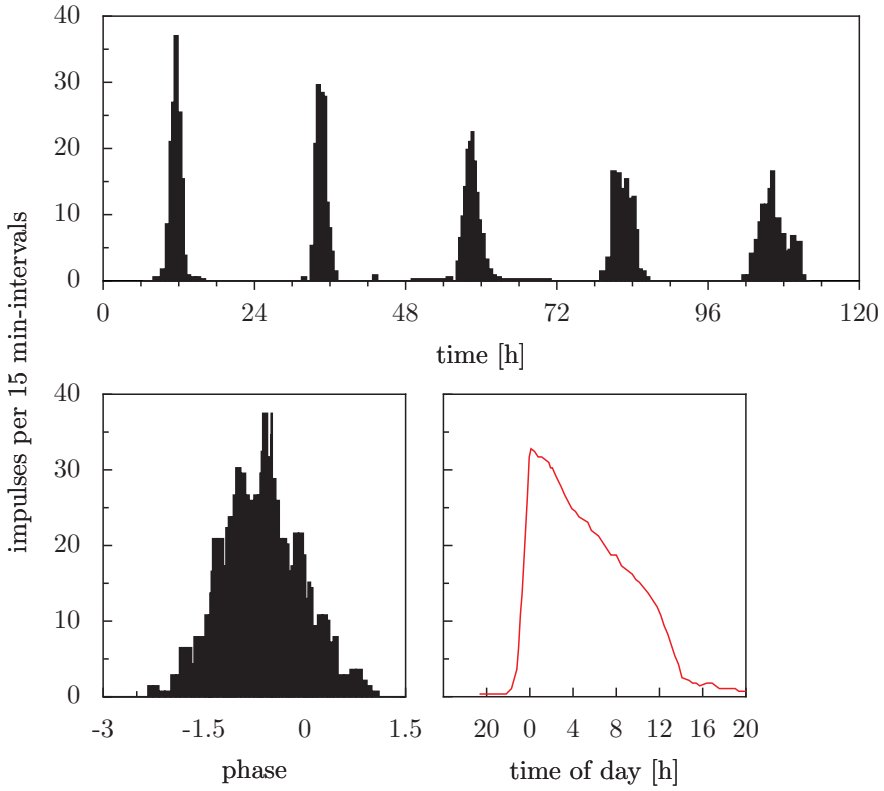

Figure 15.11: *Precision and waveform of the CAP-rhythm of isolated eyes of* Bulla *in top curve. The frequency of maxima of 377 recorded eye preparations is shown as a function of the time at which they occur. The histogram at the bottom left shows the enlarged first histogram of the top curve enlarged and in respect to the expected phase 0 of the dark-light-transistion. Bottom right: Mean waveform (red curve, see also figure 15.10) of 59 recordings of eye preparations. They consist of a rapid increase of the CAP frequency and and a slower decrease. After [119]*

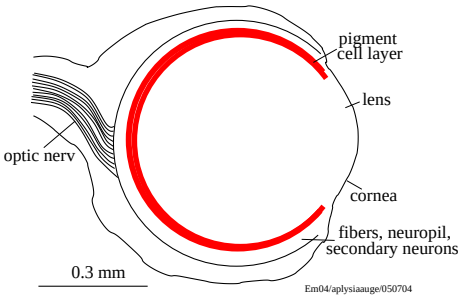

Figure 15.4: *Morphology of the* Aplysia *eye (vertical scale 0.3 mm) with simple cornea and spherical lens, layer of pigmented cells (red, several thousand photoreceptors and support cells). Next layer consists of fibers, neuropil and about 1000 secondary neurons. Fibers merge at the base and form the optic nerve, which runs to the cerebral ganglion. After [685]*

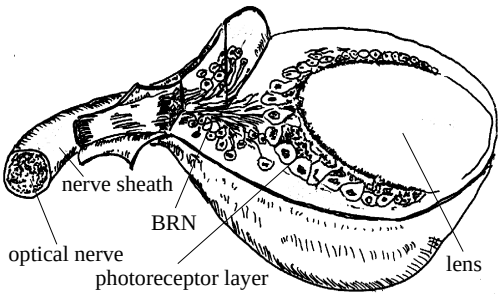

Figure 15.6: *Eye of* Bulla gouldiana. *From right to left: Lens, photoreceptor-layer, basal retinal neurons (BRN) at the base of the eye shown threedimensional; optic nerve with nerve-sheath partly cut open. After [1239]*

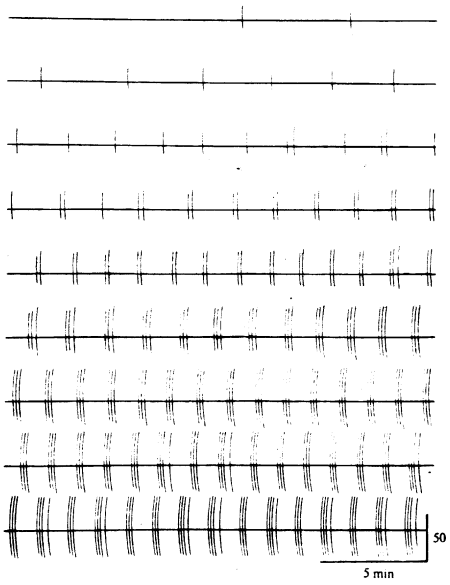

Figure 15.8: *CAP-rhythm of isolated eyes of* Aplysia *recorded in a culture medium at* 15^0C *in continuous darkness. Amplitude and frequency of the CAP vary in a circadian way. The same is true for the frequency of the CAP-bursts and the number of CAPs per burst. After [84]*

15.4 Circadian rhythm of CAP, electrophysiology

The eyes with its nerve are easy to isolate[2]. The long optic nerve (10mm) is suited for electrical recordings in organ-cultures (recording technique: tube electrode with electrical amplifier). The spontaneous activity of neurons varies periodically in a light-dark change and in continuous darkness and can be recorded as CAP (figure 15.8 and 15.9). In the morning (circadian time CT 00) the CAP-frequency is high, and low from dusk to midnight (CT 12 to CT 18). In continuous darkness the circadian rhythm continuous (figure 15.10)

[2]Surgical technique: xx

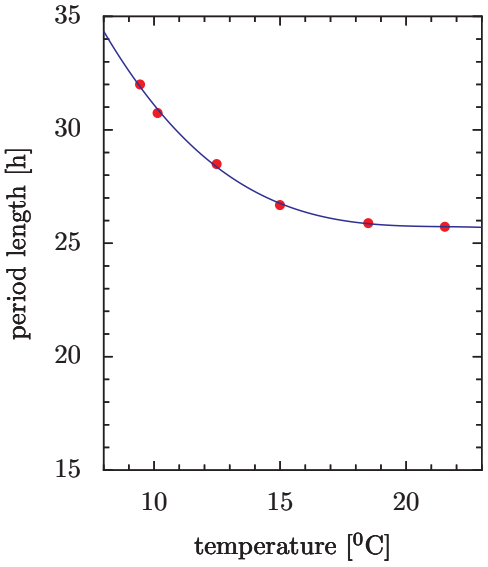

Figure 15.12: *Period length of the CAP rhythm of an optic nerve of* Aplysia *does not vary much at varying temperatures. After [84]*

and is still observable after 2 weeks in culture ([684]). Its period lenght in seawater amounts to 23-24 h (mean value of 377 records 23.74 h). In a nutrient medium it is lengthened by some of the amino acids in it by 1 to 1.7 h ([409]). The rhythm is rather precise, as shown in figure 15.11. The temperature dependency of this rhythm is low (figure 15.12). Between 15 and 22.5^{0}C the Q_{10} amounts to 1.07. Below 9^{0}C, however, temperature compensation ceases ([84]).

The CAPs are brought about by synchronous firing of the BRN-population. There are further parameter which vary in a circadian way: The CAP-burst-frequency, the amplitude (probably caused by the number of firing secondary cells), and small neuronal pulses, which are phase shifted in respect to the CAP rhythm by 180^0 and which are perhaps important for the coupling between the eyes (they are inhibited by light ([491], [625]). The small neuronal pulses are produced by retinal cells in the retina layer and are influenced by the BRN's.

The CAP-rhythms are caused by an intracellular clock: Suppression or promotion of CAPs has no influence on period length or phase. The mechanism, which is responsible for the CAP-firing, has therefore to be distinguished from the clock, which modulates the CAP-firing in a circadian way. The locomotor activity of the animals occurs parallel to the CAP rhythm.

15.4.1 Mechanism of the CAP oscillators

As far as the mechanism of the CAP oscillators is concerned, experiments were done mainly on *Bulla*. About 130 'clock neurons' (BRN) in the neuropil close to the optic nerves are responsible for the circadian rhythm. They are electrically coupled with each other.

Intracellular recordings have shown, that the membrane potential and the conductivity vary in a circadian way (figure 15.13, [1005]). During subjective day time the membrane potential is low, during the subjective night time high. In the hyperpolarized condition (subjective night time) inward directed rectifying K^+-channels, leackage channels and Cl^--channels are open (figure 15.14). These transmembran fluxes and inward directed Ca^{2+}-currents are important for events controlled in a circadian way ([980]). The circadian rhythm itself has, however, probably nothing to do with it ([766]). For the rhythm producing mechanism transcription and translation seems to play a critical role ([116], [1559]). Details are not yet known.

An important element of the cell cycle regulation, tyrosine-phosphorylation and dephosphorylation, are also decicive for

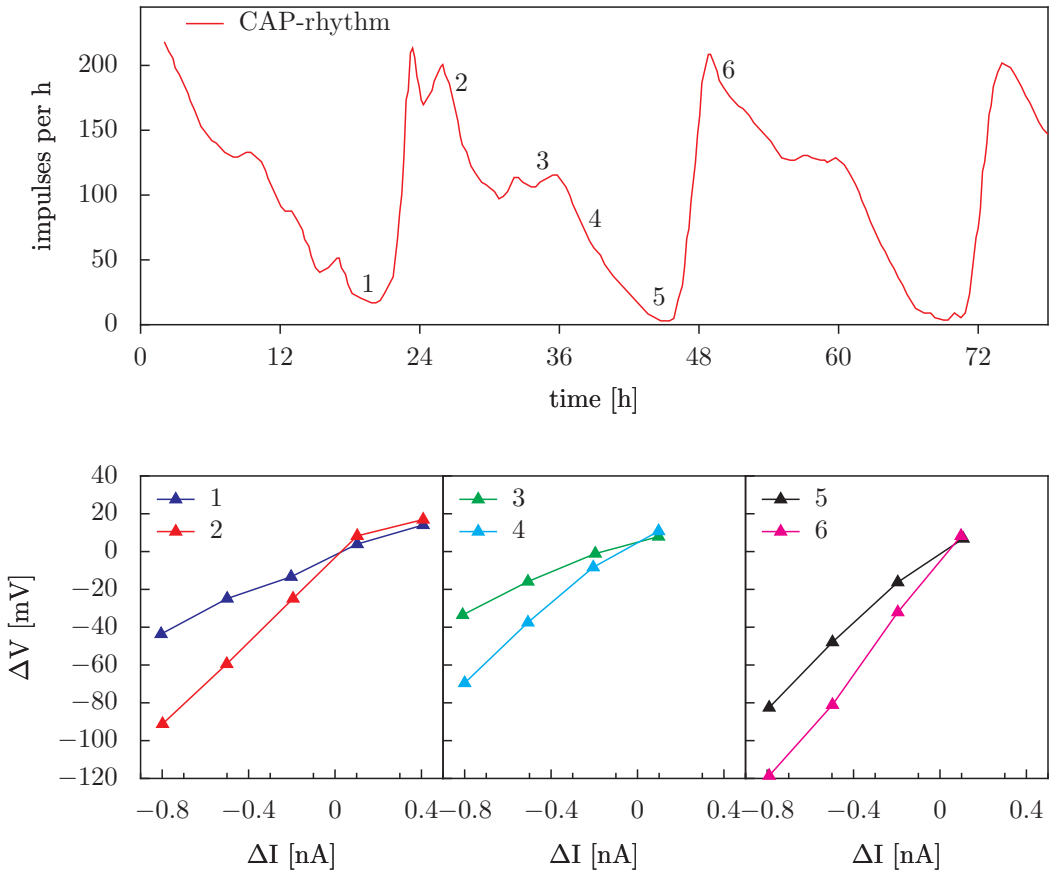

Figure 15.13: *CAP rhythm (top, red curve, impulses per hour as a function of time) and membrane-conductivity (bottom curves) of the BRN of the* Bulla *eye. Semi-intact eye preparation. Membrane potential changes recorded with current-clamp method at times, which are indicated by numbers in the uppermost diagram: Diagram (1 (blue) and 5 (black) before onset of light, 2 (red) and 6 (violet) after onset of light, 3 (green) before onset of light, 4 (cyan) after onset of light). After [1005]*

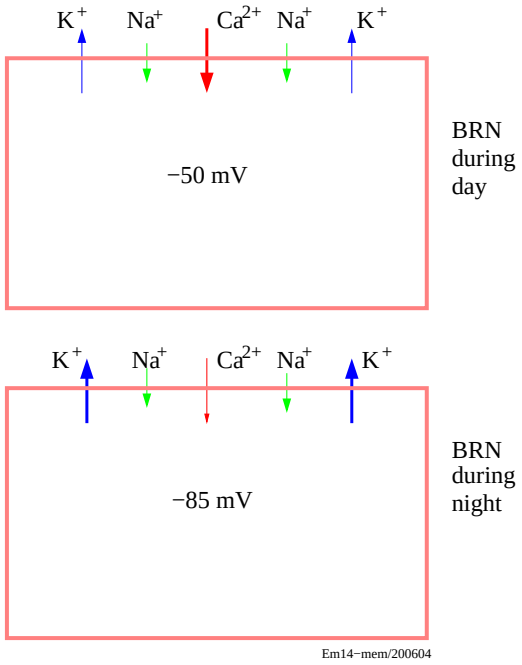

Figure 15.14: *Membrane-model of the eye-clock. During the subjective day (top cell) the pacemaker cells (BRN) in the eyes of* Bulla *are depolarized (-50mV). Action potentials are generated spontaneously, which are probably responsible for a continuous Ca^{2+}-influx. During the subjective night (lower cell) the BRNs are more hyperpolarized (-65mv). The cells do not fire. After [117]*

the circadian rhythm in the eye of *Bulla* ([1240], [1306]). Proteinkinase-activity influences K^+-channels. By this the K^+-conductivity and the membrane potential are changed ([833]).

Isolated BRNs of *Bulla* show for at least two days circadian rhythms: The spontaneous conductivity changes are hight in the late subjective night and low during subjective morning ([1005]).

In *Aplysia* monopolar neurons reacting to light were kept in culture and membrane- and action potentials recorded. They are probably the output neurons of the circadian eye clock ([683]).

15.5 Synchronisation and phase shift of the CAP-rhythm

The CAP-rhythm can be synchronized in at least two ways: A photic pathway synchronizes the oscillator to the light-dark cycle, and an efferent input allows the brain to influence the CAP-rhythm.

Light shifts the phase of the CAP-rhythm to various amounts and in different directions (advancing or delaying) depending on the time in the cycle at which it hits the circadian system. A phase response curve describes these effects (figure 15.16). The transfer from continuous light to continuous darkness determines the phase position of the CAP-rhythm.

Photoreceptors of the brain do not play a role. This was shown in experiments, in which an eye-brain-preparation was recorded in a special chamber. The eyes and the brain could be illuminated separately. Efferent fibers in the optic nerve did not pass information concerning the illumination of the eye to the brain. They did, however, activate or modulate the circadian function in the eye ([895], [1180]). That is, the efferent pathways send neurale in-

formations from the central nervous system to the eye oscillators ([823]). It is not yet known, whether the ocular pacemaker collaborate with the extra-ocular pacemaker in order to control the circadian outputs of the animal.

Light-dark cycles synchronize the retinal clock and its CAP rhythms in vivo and in vitro. Light increases the cGMP-level. In this way membranes are depolarized and calcium fluxes are induced in the pacemaker-neurons. The protein synthesis is also affected (increased, see [823]). An opsin-like protein serves as the photopigment ([130], [120], [492], [683], [490]).

15.5.1 Efferent influences of the brain

The CAP-rhythm of the eyes is not only synchronized by light, but influenced also from the brain. Efferents of the central nervous system to the eyes shift the phase of the CAP-rhythm. Serotonin (5-HT) serves as a transmitter ([254]). The cAMP-level is increased and membranes hyperpolarized by increasing the K^+-conductivity ([253]). Here too protein synthesis plays a role.

The two inputs light and serotonin merge in a common pathway: Membrane potentials of the pacemaker cells are changed via calcium (as a secondary messenger). During the subjective night the cells are hyperpolarized and inactive. During the subjective night they are depolarized. In this way phase shifts are induced and the phase of the CAP rhythm is set.

The phase shifts induced by efferent transmitters of the central nervous system (serotonin in *Aplysia*, FMRFamide in *Bulla*) are in variance from those of the light. No phase shifts (so called dead zone) are induced during the subjective night, whereas light given during this time advances or delays the phase maximally. Light has its dead zone during the subjective day. Efferent transmitters, however, advance respectively delay during the subjective day the rhythm maximally. Apparently light and efferent transmitters lead to different biochemical changes in the neurons. Light and serotonin act antagonistically. However, the interaction of the signals light and serotonin is not just subtractive at each phase, but more complex ([244]).

Serotonin modulates the behavior and physiological processes in *Aplysia*. It acts via different serotonin-receptors ([17]), which activate via the secondary messenger phospholipase C. Serotonin shifts the CAP rhythm phase dependent. It must therefore affect a component of the oscillator. This component was shown to be cAMP. cAMP activates a cAMP-dependent protein kinase ([1622]) and the K^+-conductivity is increased ([253]). The membranes are hyperpolarized ([245]).

PRC's obtained by 6 hour pulses of low Ca^{2+}-EGTA-solutions and by hyperpolarizing low Na^+/low K^+-solutions are similar. The effects do not add, if both treatments are combined. This indicates a common mechanism, through which the underlying oscillator is affected by the two treatments. Probably this common mechanism is a transmembrane-Ca^{2+} flux. It is brought about by periodic depolarizations of the membranes during the subjective day ([761]).

Since extracellular Ca^{2+} is important for synchronizing the CAP rhythm, it was checked whether it is also important for the circadian rhythm itself. This is, however, not the case ([766]).

Six hours Cl^--deprivation at different phases of the cycle advance the CAP-rhythm during late subjective night, but has only a

small (advancing) effect during late subjective day. In both cases the BRN-cells are hyperpolarized. The Cl^--effect can not be explained solely by changes in the membrane potential ([1006]).

15.6 Mechanism of the effect of light

Light acts via an intracellular cascade: It depolarizes the membrane potential of the pacemaker cells, induces a Ca^+-influx and shifts the phase of the rhythm. During early subjective night the rhythm is delayed, during late subjective night advanced (figure 15.15 and 15.16). Light is perceived by the R- and H-cells of photoreceptors (cGMP) and the signal transmitted to the D-cells of the *Aplysia*-eye (BRN in *Bulla*) (see figure 15.7). The D-cells of the eye possess a neurosecretory function and correspond to the eye stalks of Crustaceans. As neurotransmitter the catecholamine DOPA and Dopamin are used (adrenalin and noradrenalin are not found in snails). The D-cells (BRN) produce the CAP. They are transmitted to the target organs via electrical connections (gap junctions) and possibly by hormones. There they control the locomotory activity in a daily rhythm.

In continuous light the period length of the CAP rhythm is incidently one hour shorter as compared to continuous darkness. In longer continuous light the rhythm damps out. It finally stays in a condition of lowest CAP-frequency ([85]). This is the same condition which is also induced by low temperature.

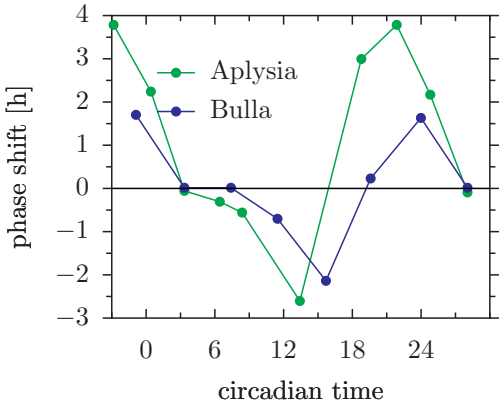

Figure 15.16: *Light pulse phase response curve for* Bulla *(blue) and* Aplysia *(green). Time of application of six hour light pulse (midpoint of pulse) on x-axis. Bulla curve after [121] and Aplysia curve after [252]*

15.7 Further circadian centres?

Since animals with removed eyes still react with their locomotory behavior to a light-dark cycle (and show freerun in continuous darkness for several days), at least one more pacemaker must exist. It is located in the cerebral ganglion, but its precise position is not yet known. For a long lasting rhythm the eyes are, however, needed.

Since the locomotor activity of animals with surgically removed eyes continues to be synchronized by light-dark cycle, further photoreceptors must be present. These extraocular photoreceptors show a broad sensitivity for wavelength. They are not yet characterized. They might be located in the mantle seam, in the abdominal ganglion, cerebral ganglion or the oral tentakels. Red light is here effective, whereas it is not affecting the CAP-rhythm.

If in the night-active *Bulla* the eyes are removed, the animal becomes day active.

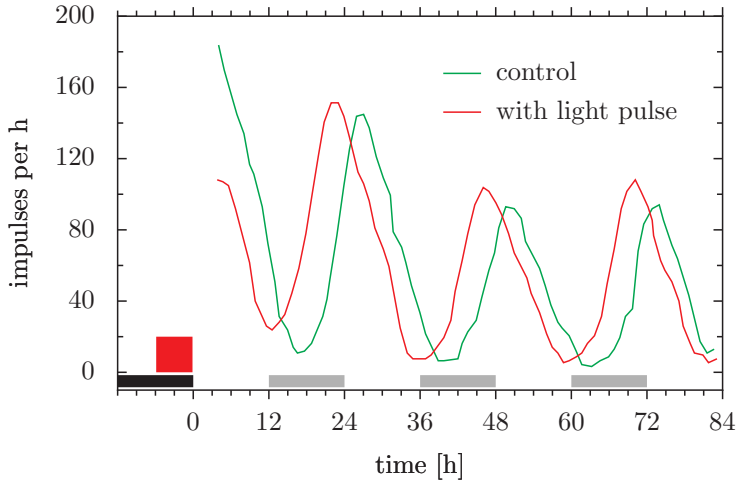

Figure 15.15: *A 6 hour light pulse (red rectangle) was given to an* Aplysia *eye preparation and the CAP of the optic nerve recorded (red curve). The other eye preparation of the same animal served as the untreated control (green curve). The phase of the red curve is advanced by the light pulse. The animal was kept in a 12:12 hour light-dark-cycle. Position of the 12 hour light period marked by grey areas, although the eyes were kept before and after illumination in continuous darkness. After [408]*

15.8 Significance of protein synthesis for the daily rhythm:

If isolated eyes of *Aplysia* and *Bulla* are treated for some hours with a translational inhibitor, the circadian clock is influenced ([686], [1595], [767]). If transcription is inhibited, the effect lasts longer as compared to the translational inhibitors (lasting the whole subjective day, [1194], [768]). These results show, that transcription and translation participate in the mechanism of the circadian feedback loop ([825]).

Experiments with translational inhibitors show ([825]): Puromycin[3], anisomycin[4] and cycloheximid[5] given as pulses all induce a phase shift, the amount and direction of which depends on the time at which they were offered ([767]). Permanently given, these inhibitors change the period length of the rhythm. General inhibitors of metabolism show the same effect as the more specific acting protein synthesis inhibitors. Apparently synthetic processes with high requirements for energy are involved. Side effects are not involved, since anisomycin derivatives without inhibiting protein synthesis do not phase shift.

But transcription is also involved in the CAP-rhythm. Critical mRNA´s are stored over night and translated in the morning into proteins. The transcription inhibitor DRB shifts the rhythm, if pre-

[3] leads to incomplete connections of the polypeptide chains

[4] inhibits transfer reaction after the aminoacyl-tRNA-formation

[5] inhibits 80s ribosomes

sented as a pulse, and increases period, if permanently offered. Putative oscillator proteins (POP's) were looked for and eight of them found. Three were characterized, which are formed after illumination as well as after serotonin application. They must somehow affect the circadian eye oscillator of *Aplysia* ([823], [824]). POP01 is a lipocorteine (Ca^{2+} phospholipid-binding protein), which inhibits the PLA-2. In another study a light- and serotonin-regulated annexin was identified in the central nervous system and the eye. It seems to participate in intracellular signal mechanisms, which finally modulate also the circadian rhythm ([756]). It was therefore assumed, that the arachidonic acid metabolism plays a role in the circadian system of the *Aplysia* eyes. It was indeed found that a LOX-inhibitor (nordihydroguaiaretic acid) phase shifts the rhythm ([1195]). A further eye-specific protein was found in *Aplysia* and an antiserum against it produced. It was used to identify the projections of photoreceptor- and pacemaker-neurons ([1427]). The chronoskeleton of the CAP-oscillator seems slowly to take shape.

15.9 Influencing circadian rhythms

Treatments, which affect either phase or period length of the oscillators, help to understand the underlying mechanism.

Low temperature given for longer time intervals shift the phase of the CAP-rhythm. Under certain conditions the rhythm can dissociate into two subpopulations, the phase of which is shifted against each other by 120^0. After some cycles the two populations of oscillators are synchronized again. Apparently they are strongly coupled with each other ([86]).

Low pH given at a citical phase (close to subjective morning) stops the CAP rhythm of *Bulla* ([765]).

Pentobarbital (an anesthesia) shifts the phase of the CAP rhythm and prevents phase shifts induced by light- and K^+. The cause of it is probably a reduced inward flux of Ca^{2+} ([763]).

The period of the CAP rhythm is shortened by inhibiting the Cl^--conductivity. Apparently the chloride channels are of significance for the circadian rhythm. Shortening of the period of circadian rhythms are so far seldom found ([764]).

LiCl (figure 15.17) and even more so RbCl lengthen the period of the *Bulla* CAP-rhythm ([762]). Continuous light lengthens also the period. Applied together period is more strongly lengthened as if each factor is applied separately ([762]). D_2O lengthens period in a concentration dependend way ([84]).

The circadian CAP-rhythm is slowed down with age of the animals. Period length as well as the phase angle increase (figure 15.18 [1392]). The proportion of transcription to translation increases with age. The period lengthening seems to be based on this and is thus the consequence of molecular biological processes of the oscillators. Older animals with opaque lenses show a damped rhythm or arrhythmia. Their retina is heavily degenerated.

15.10 Outputs of the circadian system

The activity of the pacemaker-neurons of the eyes of *Aplysia* and *Bulla* is transmitted via neurons to the brain. The signals have been detected in different connectives of the ganglia in the entire brain ([1110]).

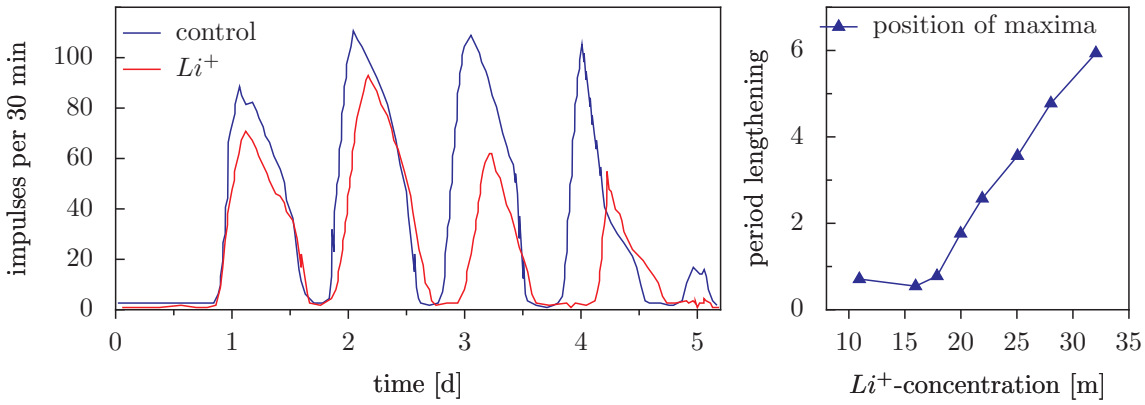

Figure 15.17: *Adding Lithium ions to the medium, in which the eyes of* Bulla *are kept, lengthens the period of the CAP rhythm (left: blue curve for control, red curve with Li^+) in a dose dependent way (right). After [762]*

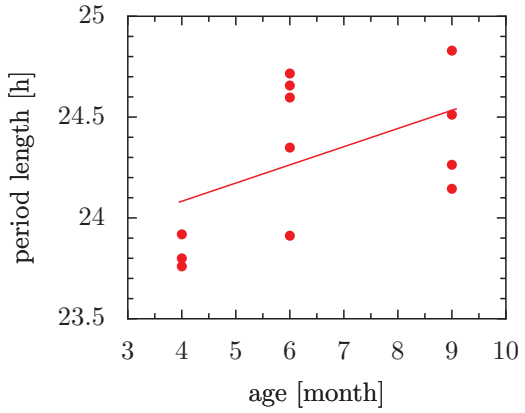

Figure 15.18: *Period length of the CAP rhythm (ordinate) as a function of age (abscissa). After [1392]*

For this purpose an antiserum for an eye-specific protein was used. It stains the target areas of the optic nerve fibers in the cerebral optical ganglion, the cerebral optical tract (synaptic exchange areas?) and the projections of the optic tract from the cerebral ganglion to the different head nerves and inter-ganglial connectives. The intensity of the immunoblot-staining shows no circadian rhythm. This protein could be involved in maintaining or regulating the retinal afferent pathways including the axons of the pacemaker-neurons ([1427]).

The resting potential is higher at night as it is during the day. In this way the excitation of the membranes of the pacemaker-neurons is changed ([1196]). The circadian clock seems to open K^+-channels in the evening, which hyperpolarize the cells. In the evening the K^+-channels close, which depolarize the cells. As a result the firing of neurons increases ([1005]). The following steps are not well enough studied so far. The BRN-cells of *Bulla* act antiphasic on spiking retinal cells ([489].

Isolated retinas of *Bulla* secret melatonin in a circadian pattern into the culture medium ([1]). In *Aplysia* a melatonin-rhythm was found in the eyes and (phase shifted to it) in the

cerebral-lobus ([1]).

It is unknown which role the circadian eye-clock plays. The visual function could be increased by it during the night ([491]), as is known from other animals and also for man.

15.11 Interactions

The circadian oscillators are housed in the pacemaker cells of the retina and possess inputs and outputs. They are, however, only a part of a larger circadian system. Efferent inputs come not only from the brain ganglia, but also from the contralateral eye.

If in a preparation with both eyes of *Bulla* the period length of the CAP rhythm of one eye is increased by Li^+ and that of the other eye decreased by Cl^--depreviation, the phase relationship of the two rhythms is not stable. This indicates a weak coupling between the two eyes. If the eye preparation is still connected with the brain, the free run period increases. Therefore efferent signals from the brain must influence the period. They might also influence the phase shift ([1124]). The coupling between the clocks in the pairs of eyes is maintained by the cerebral commissure ([1238]). As a neurotransmitter the peptid FMRF is used ([1239]).

The locomotor activity rhythm can be synchronized in a light-dark cycle (200 lux) even in eye-less animals. However, dusk is not anymore anticipated. Under constant conditions and in eye-less animals the rhythm disappears slowly, but not always completely ([896]). This indicates that there exists another pacemaker for the locomotor activity besides the one for the CAP-rhythm of the eye nerves.

The neurons of the BRN project to the cerebral- and pleural ganglia and to the abdominal connective. Many fibers cross to the contralateral ganglia. Fiber ends were found in the cerebral neuropil ([840]).

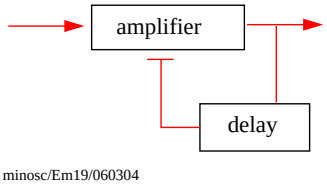

Figure 15.19: *Minimal system of an oscillator: a non-oscillating process (left arrow) is amplified and feeds back after a time delay on the process. In this way an oscillation might occur. After [457]*

15.12 Models of the circadian systems

A very simple feedback system is able to oscillate, if the parameters and the circuitry is chosen correctly (figure 15.19). First it was tried to describe the eye clock of *Aplysia* and *Bulla* with formal models. The inputs into the clock, the clock mechanism and the outputs of the clock were related to processes in the BRN (figure 15.20). After the underlying mechanisms became better known, biochemical and electrophysiological models were put forward (figure 15.21, [119]). A feedback-relaxation oscillator-model was proposed for the CAP rhythm in the *Aplysia*-eye (figure 15.23, [86]). It uses a feedback model ([889]) derived from an original proposal of Johnson/Karlsson (figure 15.22, [717]). An energy-requiring phase is followed by a passive diffusion process. The synthesis of a substance C controls the CAP frequency. The concentration of C oscillates around a reference value R. The model ex-

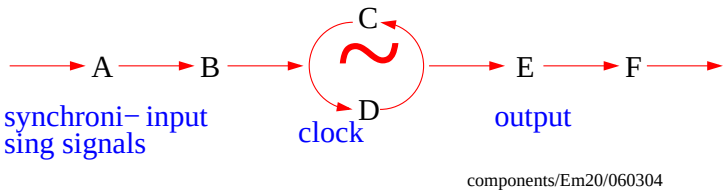

Figure 15.20: *Formal components of the circadian system: The circadian clock (in the center, C and D, ~) is synchronized by signals (intermediates A and B) with the environment (for instance by the light-dark cycle). Outputs (E and F) of the clock control processes in the cells in a circadian way. After [119]*

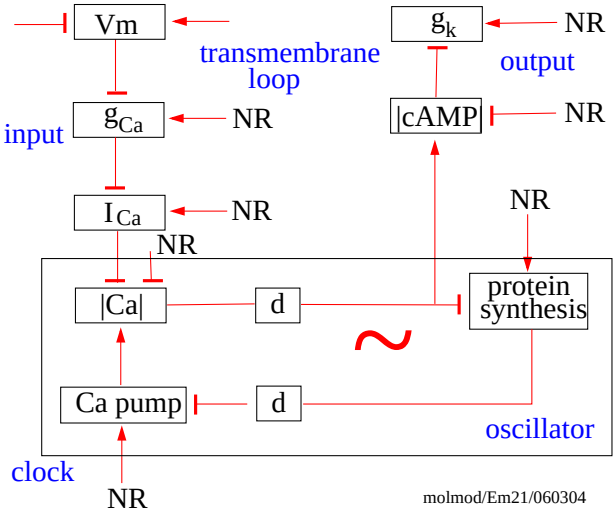

Figure 15.21: *Model to explain the rhythm and the synchronisation by light in* Bulla. *The rhythm is produced by a calcium/protein-synthesis feedback loop. Ca^{2+} leaves passively intracellular stores. By this the concentration of Ca^{2+} in the cytoplasm increases. The synthesis of critical proteins is triggered, which activate Ca^{2+}-pumps. They reduce the Ca^{2+}-level again thus completing the cycle. The elements outside the clock (violett box) represent the transmembranic synchronization-loop. Rectangular boxes are variables, squared boxes marked with d are time delays. Red lines indicate causale connections. Arrows indicate activations, lines with short strokes indicate inhibitions. NR are inputs, which do not need to be rhythmisch. After [119]*

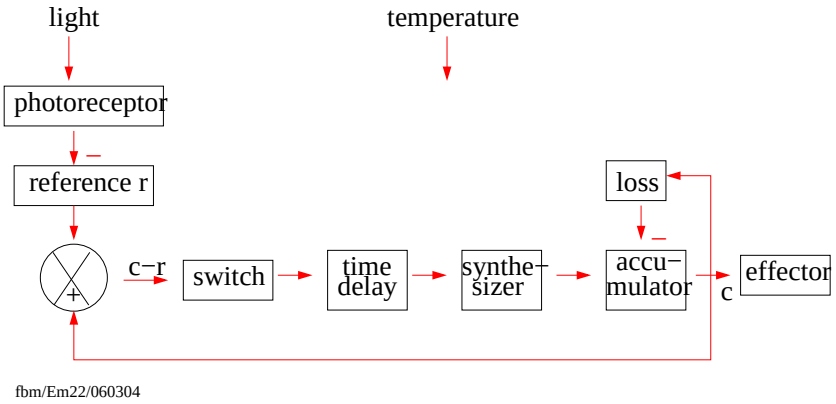

Figure 15.22: *Black-box presentation of the feedback model of the CAP rhythm: The oscillator consists of a reference value r, which is constantly compared with the output-signal c. If the concentration of c is lower than r, a switch is closed and after a certain time delay a synthetizer is activated. Its product c accumulates in an accumulator. From here the product c reaches an effector. At the same time it is fed back to a comparision unit and to a loss-function, which affects the accumulator negatively. Light is perceived via a photoreceptor and produces a signal, which reduces the reference value r (-). In the dark, r reaches its original value again. After [86]*

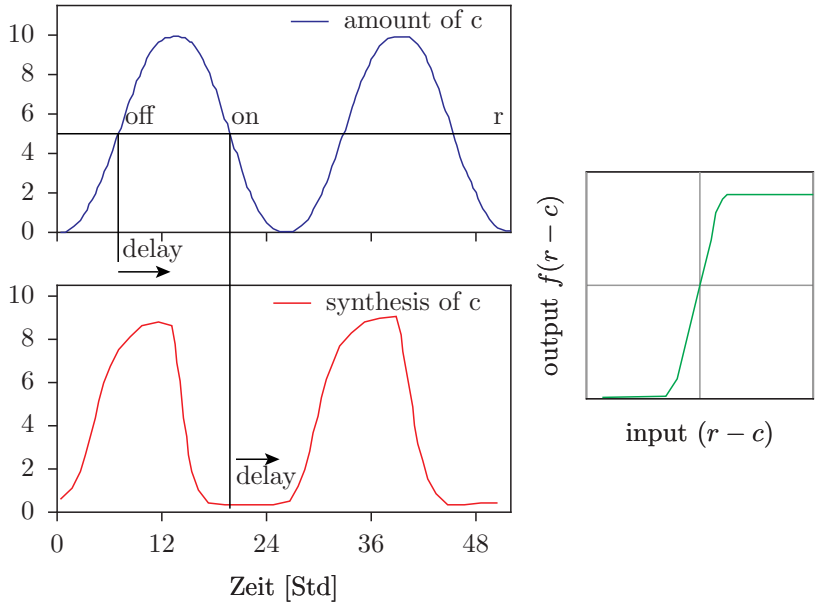

Figure 15.23: *Behavior of the feedback model for CAP rhythms (see figure 15.22). The concentration of the output element c (blue curve) is shown together with the r-value, at which the switch is activated or inactivated after a time delay (red curve). The diagram to the right (green) shows the switch-function f(c-r) as a function of (r-c). After [86]*

plains a number of experimental results with light- and cold pulses, with continuous light and heavy water. Even the dissociation into two rhythmic components can be explained.

15.13 Evolution of retinal clocks

Circadian clocks may have evolved together with light-perceiving molecules, long before photoreceptor cell and eyes specialized. Structural homologies between molecules, which are parts of the clock-mechanism, and phylogenetically old photopigments could indicate, that modern clock-proteins evolved during the course of evolution from primitive light-sensitive proteins ([269]). The function of opsins can, for instance, be modified easily by exchanging single amino acids. In this way they can adapt to the light conditions in the environment. A circadian feedback system could have evolved from primitive photopigments, which acted upon their own transcription. The photoactive yellow protein of prokaryots might serve as an example. Such develoments might have occurred several times with different photopigments as a starting point and might be the cause of the diverse clock-mechanisms, which are found among the stems of organisms.

Eyes evolved at least 40 times independently from each other during the course of the phyllogeny ([857]). It should be checked, whether primitive eyes or eye spots possess already circadian oscillators. Under vertebrates the lamprey (*Petromycon marinus*) possess already eye clocks ([995]). Lampreys diverged already 450 million years ago from other vertebrates.

An exception are the *Chelicerata* (scorpions, horseshoe crabs and spiders) and *Crustaceae*. Here the eyes do not possess circadian clocks. Instead, their function and structure is modulated by efferent signals from the optical lobes ([54], [433], [435]).

16 Circadian rhythms in *Neurospora*

In this chapter the circadian rhythms of fungi will be treated. As an example the red bread mold Neurospora crassa *is taken. It grows on a substrate and forms aerial hyphae which produce conidiospores in a daily and circadian pattern. They occur as yellow bands. The advantages of using* Neurospora *are explained. For instance, many mutants are known, several of them affecting the clockwork or its inputs and outputs. The rhythm can be influenced by light, temperature and substances. The molecular basis of the clock work and its entrainment by time cues is partly known. The circadian system seems to be more complex as known so far.*

16.1 Advantages of *Neurospora* for circadian studies

Recent studies have shown, that threadlike fungi are rather closely related to animals ([1524]). Therefore to study the circadian rhythms of them might help to understand also the mechanisms of circadian rhythms in animals. Quite a number of fungi are furthermore genetically and biochemically well studied. This is particularly the case for *Neurospora crassa*, an Ascomycet. It is a native of tropical and semitropical areas, but in the meantime has spread throughout the world. Its life cycle is shown in figure 16.1. A sexual and two asexual propagation cycles exist. In the sexual cycle ascospores are formed in asci, which are located in perithecia. In the asexual macroconidia cycle the mycelium switches periodically from a horizontal growth pattern to aerial hyphae, which produce later conidia (the pattern formation has been described by [314]; see also Genetics Stock Center's World Wide Web, http://www.fgsc.net/). This switch is controlled by a circadian clock. The period length of the rhythm is 21.6 hours in the wild type ([337]).

Neurospora crassa is well suited for studies of circadian rhythms, because it is a simple, primitive eukaryotic organism, which can be easily reared. In liquid cultures it produces large amounts of mycelia, which can and have been used extensively for biochemical studies. The fungus is haploid, can be crossed easily, has been studied genetically, biochemically, and physiologically very intensively and many mutants exist. The generation time is short. This fungus is also open to molecular biological studies and has been worked on in this respect a lot. The formation of asexual conidia serves as a hand of the circadian clock which can be measured simply. For *Neurospora* as a research object see [1150].

An older review article covering earlier studies on circadian rhythms is by [1582] and later reviews are from [420], [421], [423], [850], [78], [1224], [1057], [921], [846].

The processes under circadian control are for the different organisms quite divers and special. The mechanism of the circa-

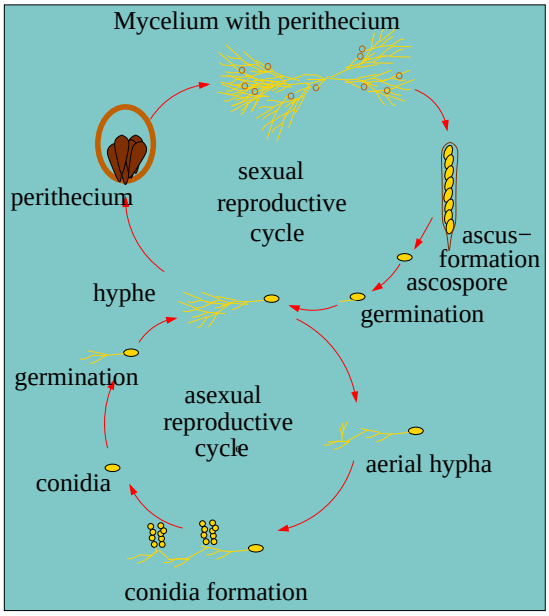

Figure 16.1: *Developmental and generation cycle of* Neurospora crassa. *Top: Sexual reproductive cycle. Ascospores are formed in asci, which are located in perithecia. After germination an ascospore forms a mycelium (coenocytic, that is, many nuclei share a common cytoplasm). Via properithecia perithecia are formed. In a perithecium asci are produced from which the ascospores are dispersed which form mycelia again. The asexual cycle of propagation has closed. Bottom: Asexual reproductive cycle. The mycelium might produce also asexual conidia ('macroconidia'): Aerial hyphae are formed, which produce later conidia. They germinate and form new mycelia (the pattern formation of* Neurospora crassa *was described by [314]). The switch between undifferentiated mycelia and aerial hyphae is controlled by a circadian clock. After [1404]*

dian clock could, however, follow a general principle. If the clock of *Neurospora crassa* would be understood, decisive hints for other circadian controls could be given. Of all circadian systems the circadian clock of this fungus is quite well known. It might be one of the first eukaryotic organisms, in which the inputs to the clock, the mechanism of the clock and the outputs of the clock to the controlling processes will be completely understood in molecular terms. For reaching this aim it is helpful that the genome of *Neurospora crassa* is completely known.

16.2 Circadian rhythms of conidiation and other events

Conidiation in *Neurospora crassa* is controlled by the circadian clock. Under daily cycles of light and darkness these asexual spores are produced in the late night and early morning. During conidiation a dense mycelium is formed. It is more branched as compared to the normal mycelium and aerial hyphae grow out of the medium. Conidia will form on the tips of these hyphae (figure 16.2). Macroscopically they are recognizable as yellow-red bands. Since growth is constant in spite of conidiation[1], the rhythm can be determined in a growth tube simply by using a ruler and by determining the distance between the centers of sequential

[1] see, however, [851]: By using time lapse video imaging, it was found that the band/interband determination at the growth front is not tightly coupled to the subsequent synchronization of conidiospore differentiation. This might indicate the existence of two circadian oscillators controlling these two events and implies also that growth is not always constant.

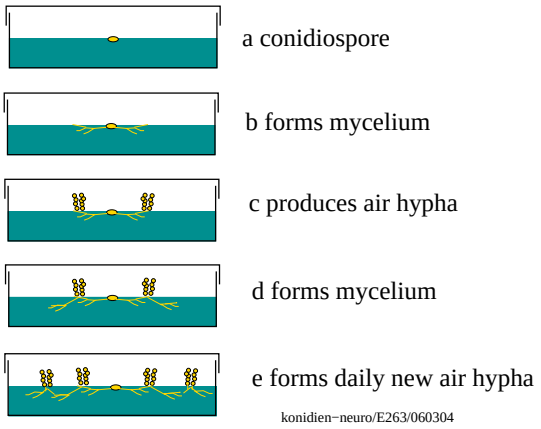

Figure 16.2: *Conidiospores of* Neurospora crassa *germinate (a) and grow as hyphae on a substrate to form a mycelium (b). After some time aerial hyphae are produced which grow out of the substrate into conidiophores, which form conidiospores (c). Growth along the substrate continues with normal mycelium after conidiation, until the next conidial band is formed (d). This occurs daily. After [1223]*

conidia bands (see growth tube in figure 16.3 and a video of growing *Neurospora crassa* with protoplasm streaming from the Genetics Stock Center's World Wide Web, http://www.fgsc.net/.

A special mutant, bd, is used which produces conidia also in closed tubes, because in the wild type the accumulating CO_2 suppresses conidiation. The growth of the strain bd is furthermore reduced by 30% compared to the wild type[2]. In most cases a further mutation is crossed in, csp. This mutant does not form a septum between conidia and aerial hyphae. The conidia will therefore stay attached to the aerial hyphae. This prevents spreading of the conidia to other parts of the growth tube (or other laboratories).

If the phases between two parts of neighboring mycelia differ from each other, the difference in phase is maintained even after contact of the mycelia. This indicates that the circadian oscillator in the mycelium is locally autonomous. This finding is confirmed by transferring pieces of mycelium into growth tubes ([1151]). Old mycelium exhibits also a circadian rhythm ([320]).

At constant temperature and in physiological darkness (weak red light) the free run period is 21 to 22 hours. The rhythm is temperature compensated between 18^0 and 30^0C. It can be phase shifted by light and temperature pulses.

The conidiation of *Neurospora* can be used for experiments in courses and in schools ([387] and [313]). See also [380] under 'Experiments in schools' (the book is available in the Internet (http://w210.ub.uni-tuebingen.de/volltexte/2009/3790/).

Conidiation is controlled by the circadian oscillator, but the oscillator runs also without conidiation. In this case the running of the oscillator can be seen by for instance biochemical rhythms. Examples are the amount and synthesis of nucleic acids, protein content, activity of enzymes ([640]), the ratio between ADP and ATP in the mitochondria[3]. Furthermore, some fatty acids of membranes fluctuate in a circadian pattern (see page 372).

Other events connected with differentiation are under circadian control. The signals which control them and metabolic processes underlying them are not yet known. cAMP might be such a signal which fluctuates in a circadian way ([584]). The circadian clock of *Neurospora* can be observed also in single cells (fresh germinated conidiospores) ([903]).

[2] biotin-containing carboxylase complex might be affected in this mutant.

[3] energy charge $(ATP + 1/2ADP)/\sum(ATP + ADP + AMP)$, [304], [1339]

16.2.1 Liquid cultures

For biochemical studies of circadian rhythms large quantities of mycelium from different phases are needed. To harvest parts of the mycelium growing on agar medium in dishes is unsuited. Fortunately liquid cultures of *Neurospora crassa* display also circadian rhythms ([1151]). A special method was used to disentangle growth and rhythmicity: A panthotenic acid deficient mutant was grown on a liquid medium containing panthothenic acid, until mats of the mycelium had formed on the surface. Circular pieces of these mats were obtained using a cork borer and transferred to liquid cultures *without* panthothenic acid. Thus they where unable to grow from now on. In spite of it the circadian rhythm continued: If the pieces of mycelium were transferred to the agar of a growth tube containing panthotenic acid, circadian banding was observed (figure 16.3). The phases differ if the transfer is done at different parts of the circadian cycle. In these liquid cultures without panthotenic acid the effect of inhibitors on the rhythm can be studied without the danger, that the effect is the result of inhibition of growth (since growth is suspended, see [850]).

The circadian clock of Neurospora can be observed also in single cells (recently germinated conidiospores) ([903]).

16.3 How light influences the conidiation rhythm

The most important environmental factors influencing circadian rhythms are light and temperature. Both were studied intensively in *Neurospora crassa*. Usually the light-dark cycle is the most prominent time cue for synchronizing circadian rhythms. However, in *Neurospora* it turned out that temperature cycles are more important and override the entraining effect of light-dark cycles if given simultaneously with them ([906]).

How light influences the circadian rhythm of *Neurospora crassa* is treated in an older review of [1308]. More recent reviews are by [423], [1226], [460], [850] and [902]. In connection with other systems light effects are reviewed by [1090] and [362]. [734] discusses the recent situation. The molecular basis of light effects on the circadian oscillator in *Neurospora crassa* was studied by [270]. The results are compiled by [343]. The light effects have also been modeled recently in respect to the molecular basis of the clock mechanism ([1284]).

Light pulses: Light pulses shift the circadian rhythm of conidiation in *Neurospora crassa* ([1309]). Experiments with two short light pulses given behind each other show that the circadian oscillator is shifted already 45 minutes later ([320]). Direction and strength of the shift depends on the time in the cycle at which the light pulse was administered. A phase response curve to light pulses shows these shifts (figure 16.4, [1308], [320]). Depending on the strength of the light pulse a strong or a weak phase response curve (see figure 14.6 for an example in *Drosophila*) is obtained. The rhythm is shifted already to its full extent by the light pulse in the same cycle it was applied. In circadian rhythms of other organisms there are usually transients observed until the final shift is reached after several cycles.[4]

[4] The phase shifts by light pulses can be prevented by DES, which is an ATPase-inhibitor acting in

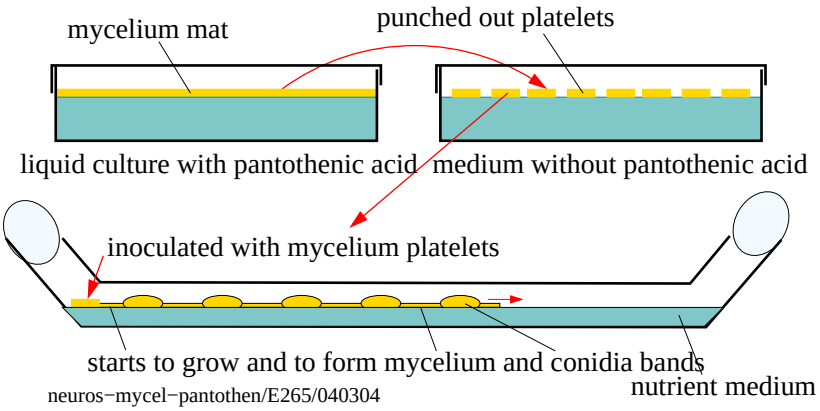

Figure 16.3: *Circadian rhythms occur also in liquid cultures of* Neurospora crassa. *A panthotenic acid deficient mutant was grown in a medium containing panthotenic acid until on the surface mats of mycelium had formed (top left). With a cork borer circular pieces were cut out and transferred in liquid cultures without panthotenic acid (top right). Therefore no growth can occur. In spite of it the circadian rhythm continues: Circadian bands are formed on agar of a growth tube containing panthotenic acid and thus allowing growth again (bottom)*

How long does it take to shift the underlying oscillator by a light pulse? To answer this question, experiments using two successive short light pulses were done and the time between them varied. If the first pulse has shifted the clock, the phase shifting effect of the second pulse should be predictable on the basis of the phase response curve of the first pulse. [320] showed that after 60 minutes the circadian oscillator had been shifted by the first pulse. If the second pulse followed the first one in a shorter time, a biphasic response was observed. This nontrivial result could be simulated by [1284] in assuming different states for the light signal transduction pathway.

The amount of phase shift depends on the environmental temperature: At higher temperatures the phase shifts by light are smaller.

DES as an ATPase inhibitor prevents phase shifting by light. Venturicin and oligomycin have no effect: They inhibit only the mitochonrial ATPase. Azide inhibits both, plasmalemma and mitochondrial ATPase and prevents phase shifting by light.

Light-dark-cycles: *Neurospora crassa* can be synchronized to a 12:12 hour light-dark-cycle. Shortly before light-on the conidial band starts being formed and is finished some hours after light-on (figure 16.5). However, five minutes of light given ones per day are already sufficient for synchronization.

The range of entrainment can be determined by using light-dark cycles shorter or longer than 24 hours (for instance 11:11, 13:13 hours light-dark). It is quite large and depends, as expected, on the light intensity ([864]).

the plasmalemma. Venturicin and oligomycin, which inhibit mitochondrial ATPases, do not inhibit it. Azide inhibits both plasmalemma- and mitochondrial ATPase and prevents phase shifting by light.

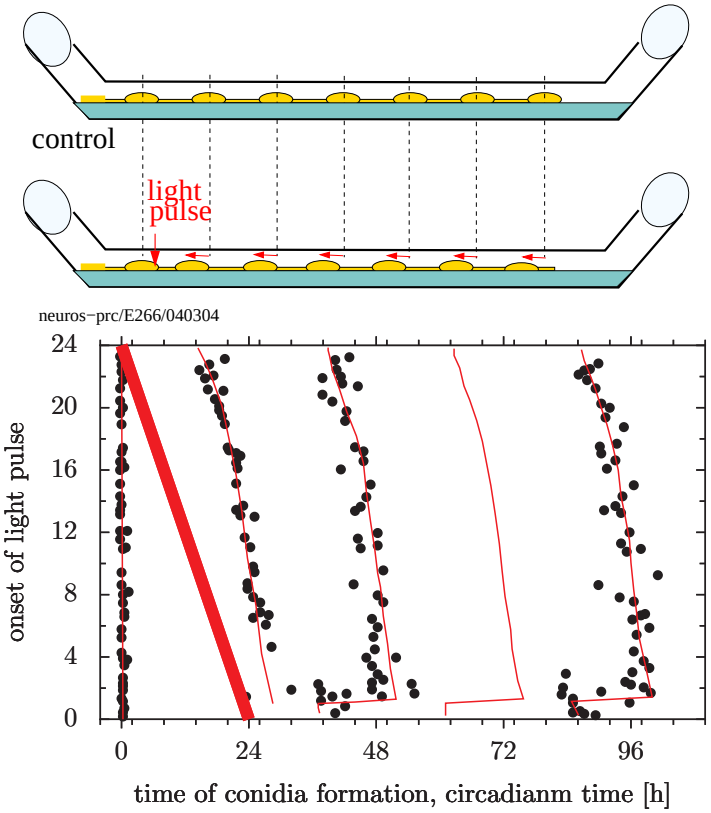

Figure 16.4: *Phase responses of rhythmic conidiation of* Neurospora crassa *to single 45 minute light pulses of 14 000 Lux. Experiments as shown in the upper part of the figure show phase shifts of the conidiation bands due to light pulses. If administered at different phases of the cycle (as shown by the red inclined lines and indicated at the y-axis in the lower graph), the centers of conidiation bands (dots, fitted by red curves) in respect to untreated controls (their centers of conidiation bands are represented by the thin vertical lines through 24, 48, 72 and 96 hours) are in variance with those of the controls. Phase advances (centers of bands earlier than control bands, upper part of diagram) and delays (centers of bands later than control bands, lower part of diagram) are found, and a phase jump from strong delays to strong advances at times where the light pulse begins around 7:00. First, second, third and fourth bands are shown. Since period length is about 22 hours, the time on the x-axis is given in subjective time (24, 48, 72, 96 hours instead of 22, 44, 66, 88 hours real time). After [1308]*

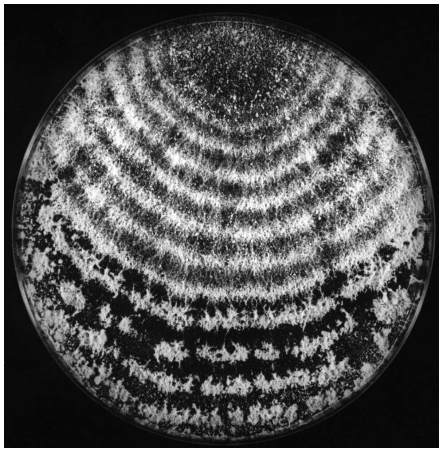

Figure 16.5: *Conidiospores were inoculated at the agar medium and they started to grow first under continuous darkness. Synchronization of the circadian conidiation in* Neurospora crassa *occurred after transfer to a 12:12 hour light-dark-cycle. Shortly before light-on the conidiation bands begin to be formed and end several hours after light on*

Continuous light: In continuous light of sufficient intensity the conidia are not formed rhythmically any more. Therefore no bands can be seen (figure 16.6). Light intensities of 4.2erg/cm^{2}sec are already sufficient to suppress banding. Probably the continuous light prevents rhythmic conidiation without stopping the oscillator ([1126]). In poky, a mitochondrial mutant, much more light, namely at least 23000erg/cm^{2}sec is needed for preventing rhythmic conidiation, and in an albino mutant, as little as 210erg/cm^{2}sec is sufficient.

Is the same pigment used for suppressing rhythmic conidiation and for phase shifting the rhythm by single light pulses? [1126] selected mutants which continue to show circadian conidiation even under continuous light. Although the rhythm of conidiation is less sensitive to continuous light, phase shifting, synchronization and carotenoid synthesis are normal. This indicates, that rhythmic conidiation is suppressed by a special photoreceptor, or the signals are transmitted via different pathways. Blind for all kinds of light effects are the wc mutants (page 16.8.1 and [1289], see however [661]).

Photoreceptors: Light is perceived by photoreceptors. An intracellular signal is produced and transmitted to the circadian clock. There it influences the size or activity of a state variable of the oscillator.[5]

Different organisms often use various pigments for synchronizing circadian rhythms. As mentioned before and more detailed described for *Drosophila* (subsection 14.2.4), the circadian clock of one species is usually synchronized by the use of several photoreceptors. Even in a unicellular organism different photoreceptors have been found ([1250]). In *Neurospora* only blue light seems to affect the clock both in phase shifting as a pulse as in damping the rhythm by continuous light (maximum around 465 nm, figure 16.7, review by [943]). UV light shifts also the phase. But for other light responses additional photo-pigments might be involved.

The light is perceived by flavin containing substances. One or several flavin-type photoreceptors might participate. During light perception free cellular flavin in the triplet state is used for electron transfer ([460]), or a flavin bound to cytochrome b557 in the singlet-state ([1401]).

Carotenoids ([1288])[6] and nitrate reduc-

[5] A state variable is an essential component of the clock. It is not sufficient, that this component is present in the cell. It has also to fluctuate rhythmically in order to keep the clock running.

[6] This is shown by experiments with a mutant, in which three albino-mutations al-1, al-2 and al-3

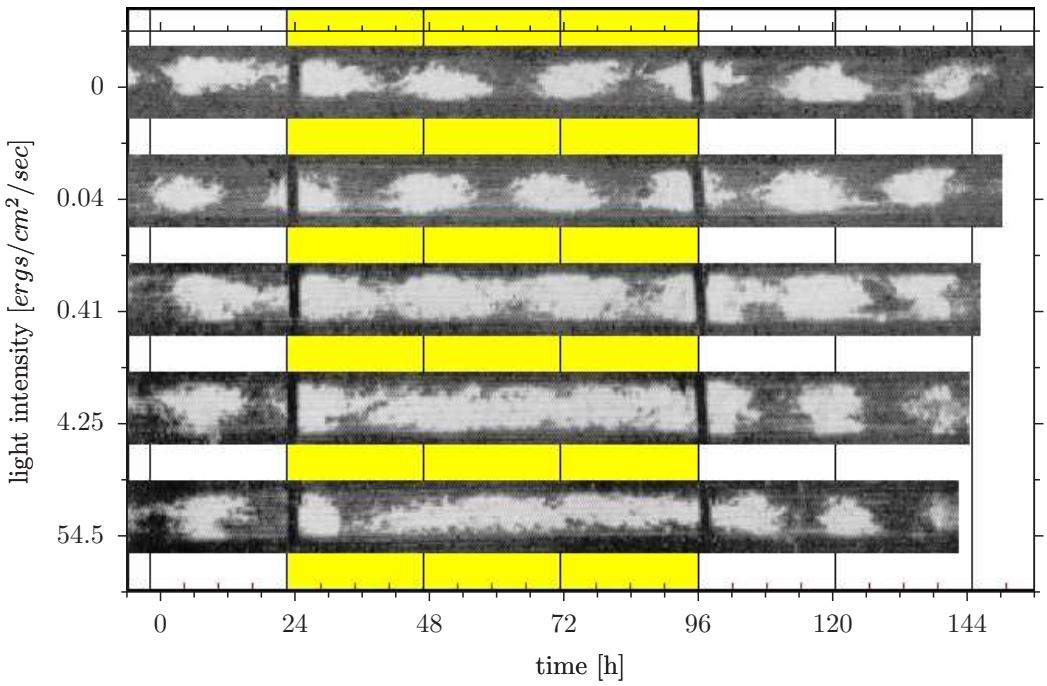

Figure 16.6: *Growth tubes containing mycelia of* Neurospora *were transferred from the dark for 72 hours (yellow background) in continuous light of varying intensities (as shown on the y-axis). The rhythmic formation of conidia is suppressed already by* 4.2 *sec. After [1126]*

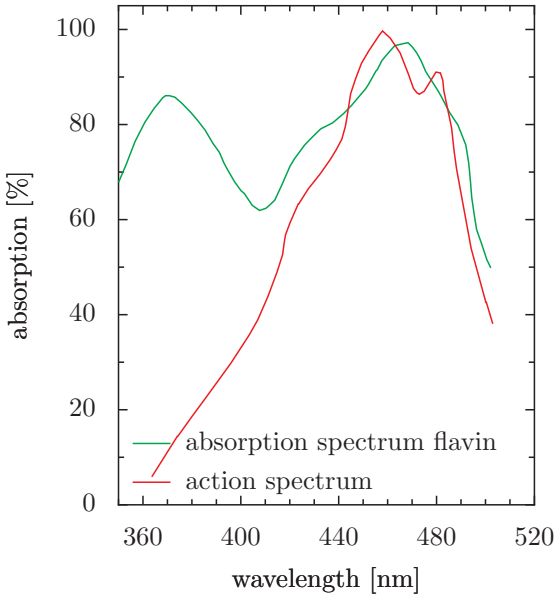

Figure 16.7: *Action spectrum of rhythmic conidiation in* Neurospora crassa *(red) and absorption spectrum of flavin (green). After [63]*

tase, a flavin containing substance, are not involved in light perception ([1091]). Nor is cryptochrome, although CRY1 is present in *Neurospora*. It does not function as a photoreceptor in carotenogenesis and in light regulated transcription.

are combined. The carotenoid content was less than 0.5% of the wild type ([1288]). Likewise, the poky-mutant reacts less strongly to light. This mutant does not produce cytochrome ([148], [1339]). Riboflavin-deficient mutants (rib) show a reduced reaction toward light ([1125]). Absorption spectra speak also in favor of a reduction of a cytochrome b by flavin. Cytochromes of different origin (plasmalemma, mitochondria, endoplasmatic reticulum) might play different roles. The cytochrome in the plasmalemma could be responsible for the influence of light on the rhythm ([131]). Light blind mutants wc-1 and wc-2 are still synchronizable. The induction of the frq by light is, however in this mutant prevented ([919]). Strong blue light bleaches the photoreceptors irreversibly ([1047]).

The blue light photoreceptor seems to be close to the clock mechanism. But it is apparently not an integral part of the circadian oscillator of *Neurospora crassa:* It can be separated from the clock by disrupting light input pathways (in mutations) without preventing the circadian clock from running. Into which kind of signal (signals?) the light is transformed after its perception and how this signal affects the circadian clock is studied intensively ([943]). Mutants are used for this purpose, in which the sensitivity toward phase shifting light is changed (poky, rib-1, rib-2, wc-1, wc-2). Furthermore substances are used, which block the transduction of the light signal (azid, DES, DCCD, ethanol, overview in [850]).

From other systems it is known that one of the following four mechanisms is responsible for the transduction of light signals:

1. Activation of the phospholipase C, IP_3 is produced, Ca mobilized and protein kinase C activated.
2. cAMP- and/or cGMP-activities are influenced
3. The protein kinase activity of the receptor is modulated
4. Ion channels are modulated.

It seems that mechanism 3 holds for *Neurospora*. Blue light activates WC-1 rapidly by phosphorylating it via a protein kinase C, a light specific, positively acting element. WC-1 is thus the substrate for protein kinase C ([943]). It is possible, that in this way the FRQ protein is phosphorylated. It contains three functionally important phosphorylation sites for phosphokinases. The kinases are redundant, but most of them are calcium/calmodulin

dependent ([1592]). The PER protein of *Drosophila* is also known to exhibit circadian fluctuations in phosphorylation. The double mutant white color wc-1 and wc-2 is blind ([1289]). The role of WC in entraining and phase shifting the circadian oscillator in *Neurospora* by light is discussed together with the molecular mechanism in section 16.8.

16.4 Temperature effects and temperature-compensation

Besides light pulses temperature-pulses are also able to shift the circadian rhythm of *Neurospora crassa*. In fact, in this species temperature cycles seem to be a stronger time cue as compared to light-dark cycles ([906]). Depending on the strength and duration of the temperature pulse, strong and weak reactions are evoked. These effects are found in cultures on agar and in liquid cultures. Heat pulses are more effective as compared to cold pulses. Already a difference of 2^0 given once every 24 hours synchronizes the rhythm of *Neurospora crassa* ([452]).

A proper clock should at different environmental temperatures have the same speed. It is assumed, that this applies also to circadian clocks. But this assumption has never been tested. Temperature compensation could also be just a side effect, brought about for instance by the clock mechanism and without adaptive significance. Thus, even homeothermic animals possess a temperature-compensated circadian clock ([493]). On the other hand there are also 'circadian' rhythms with a poor (*Phaseolus*, [973]) or a lacking temperature compensation (*Thallasomyxa australis*, [1383]).

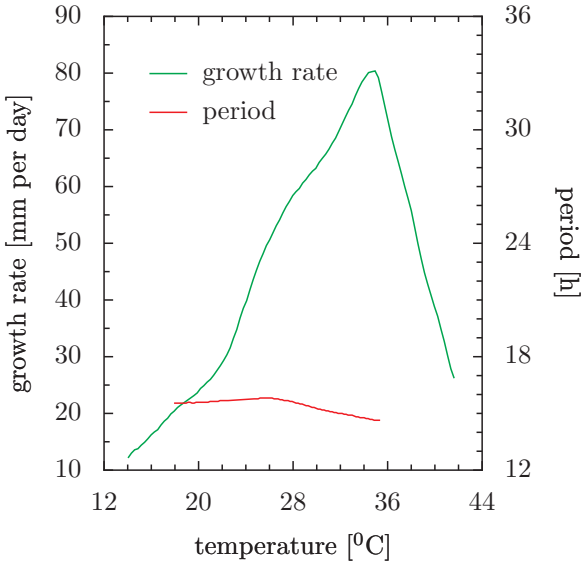

Figure 16.8: *The circadian conidiation of* Neurospora crassa *is temperature compensated between* 18^0 *and* 30^0 *(red curve, right y-axis), whereas growth rate depends strongly on temperature (green curve, left y-axis). After [476]*

The circadian conidiation in *Neurospora crassa* is temperature compensated between 18° and 30° only ($Q_{10} = 0.95$, figure 16.8, [476]). In other organisms temperature compensation of the circadian clock is also restricted to certain limits. Outside of the 'allowed temperature range' temperature compensation does not exist or the circadian clock stops and stays at a certain phase of the circadian cycle.

At higher temperatures the Q_{10} is 1.3, and the period becomes medium-dependent. The mutants frq 1, 2, 4 and 6 with period lengths shorter than the wild type possess also this 'breakpoint' at 30°C. In the mutants frq 3, 7 and 8 with longer periods as compared to the wild type the breakpoint is lower and the periods of frq 7 and 8 are not temperature-compensated beyond 22°C. The long period is, however, not in itself the cause of the lost tem-

perature compensation, because in the mutant chr with a long period temperature compensation is also found beyond 30° and in prd 3 the Q_{10} for temperatures below 30°C is smaller than 1. prd 4 with a shorter period possesses several breakpoints. Finally, chr*prd has below 27°C an unusual low Q_{10} of 0.86.

Temperature compensation is completely lost in frq 9. The Q_{10} is 2.0 and thus of the same size as growth rate ([922]). The rhythm is, however, not just coupled to the growth rate, since period length depends on the composition of the growth medium in a different way as growth rate does. The results speak in favor of frq-locus being directly responsible for temperature compensation. On the other hand temperature compensation in *Neurospora crassa* is not necessarily needed for the occurrence of the rhythm.

The *cel* mutant has lost its temperature compensation of the circadian rhythm below 22°C ([969]). *cel* has a defective fatty acid synthetase complex. If fatty acids are added, for instance palmitic acid, *cel* grows normal and the period lengths is temperature-compensated again. Thus membranes seem to be involved in the circadian rhythm of *Neurospora crassa* and the homeostasis of membrane fluidity has been assumed to be responsible for temperature compensation (see, however, the discussion below and figure 16.16).

Different models were put forward to explain the temperature compensation ([1282], [390]):

1. According to [341] a reaction A $\Longrightarrow$ B proceeds faster at higher temperature. This reaction is inhibited by the product of a second reaction C $\Longrightarrow$ D, and the inhibition is stronger at higher temperatures. At temperatures beyond 30°C inhibition is not sufficient anymore and the temperature compensation is therefore not met. In frq 1 the process C $\Longrightarrow$ D is speeded up. Therefore more D is produced and temperature compensation affected (figure 16.9). At 30°C temperature compensation is lost, since the rate of inhibition by D can not be increased anymore. The rates of critical reactions have thus to be in certain limits for temperature compensation. If mutations affect these reaction rates, temperature dependencies are changed. In frq7 D might be overexpressed or it is more stable. Therefore at all temperatures more D_2 is available, the reaction A $\Rightarrow$B is inhibited at all temperatures and is thus slowed. Period length of this mutant is larger and temperature compensation affected.

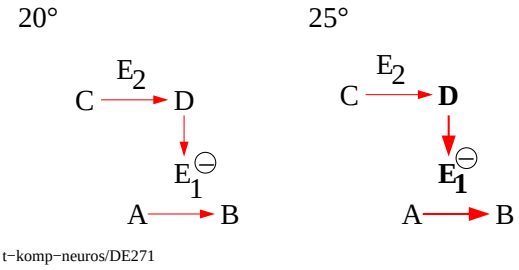

Figure 16.9: *Model to explain temperature compensation of circadian rhythm of conidiation in* Neurospora crassa *by two opposing reactions, both of which depend in their reaction rate on temperature. Due to inhibition of the enzyme E1 (A $\Rightarrow$B) by D the period is kept constant. After [341] based on a proposal of [581]*

2. The biochemical oscillator model of [1144] includes activation and inactivation of an enzyme. Temperature compensation requires temperature independent ratios of rate constants. Certain rate constants are diffusion controlled and therefore temperature independent. The product between rate constant and steady state concentration of an enzyme species has

to be temperature independent. The mechanism of this temperature compensation is apparently based on an temperature-induced conformational change of proteins ([1398]).

3. According to another model the fatty acid composition of the membrane is responsible for the temperature compensation ([265]). The cel mutant with changed fatty acid composition of the membranes has lost its temperature compensation of the circadian rhythm, as mentioned already. For details see [849] and [845].

4. An antagonistic balance of chemical oscillators leads according to Ruoff (reviewed in [1282]) to temperature compensation, because period-increasing and period-decreasing reactions are component processes. If based on the Goodwin oscillator model ([511]) with negative feedback, phase responses to temperature steps and temperature pulses are correctly predicted and temperature compensation holds. The degradation reactions influence period length, whereas synthesis rate constants do not. Clock proteins, clock mRNA and transcriptional inhibitors are simulated correctly (see figure 16.15 and 16.11)

5. A further explanation for the temperature compensation results from a model of [848] for the circadian rhythm of *Neurospora crassa.* According to this model, the amplitude of the oscillation plays an important role. It was observed, that mutants with prolonged periods react less strongly to light pulses and cycloheximid-pulses as compared to the wild type. The authors explain it by the size of the amplitude. If it is larger, the same pulses lead to a small phase response. Temperature compensation is explained in the following way: With increasing temperature the amplitude becomes larger. Thus the path on the limit cycle increases, but the process runs also faster: As a consequence, the period stays constant (figure 16.10).

6. Recent results of molecular biological studies led to further hypotheses, which might explain the temperature compensation of the circadian clock in *Neurospora crassa* ([905], see page 378).

16.5 Effects of substances on the circadian rhythms

In the preceding section the relatively small influence of environmental temperature on the circadian clock of *Neurospora* was described. Circadian clocks are, however, also rather insensitive against other influences such as metabolism and all kinds of chemical compounds. This holds for *Neurospora*, although perhaps to a lesser degree as compared to other organisms in which the influences on circadian clocks were studied. Some examples are given in the following.

The culture medium influences as a rule the period length only little. With acetate, casamino acids and Vogel salt solutions the bands are more clearly expressed. Different amino acids such as arginine, tryptophane, histidine and alanin influence the rhythm, others (lysine, cystein, glycin, methionin, thyrosin) not. Some amino acids stimulate conidiation and the rhythm continues for a longer time. The growth media influence only the hand of the clock,

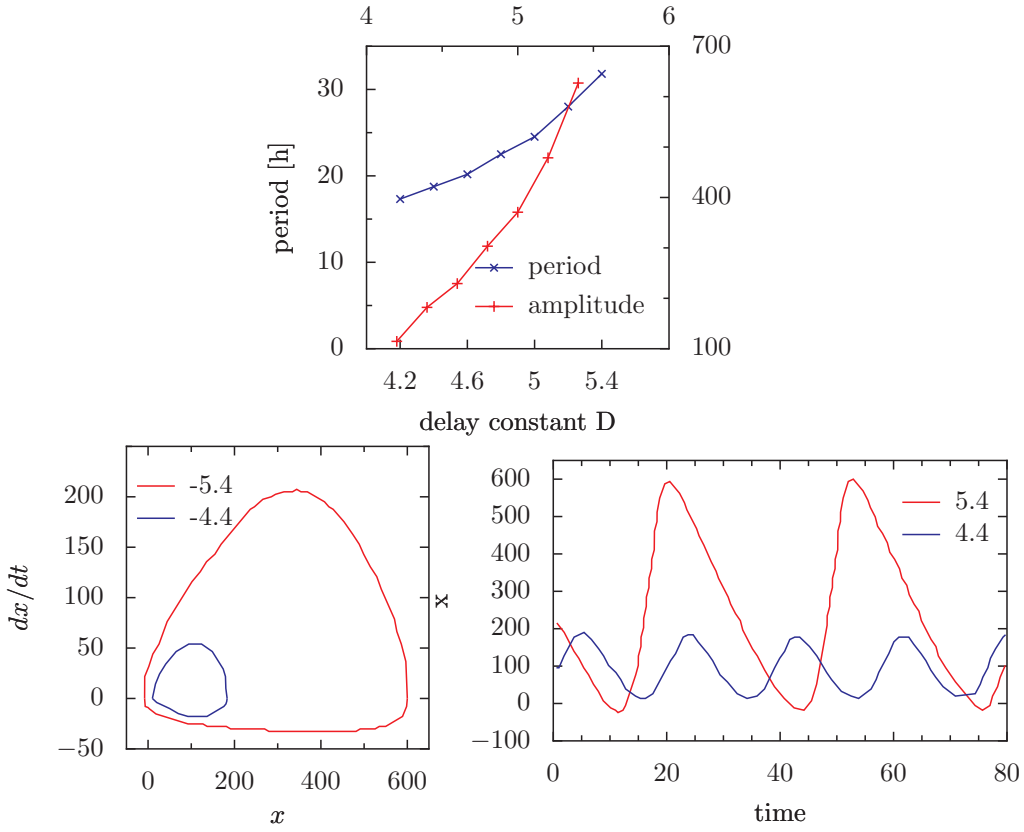

Figure 16.10: *Temperature compensation by amplitude changes of a time-delay oscillator. Top: Change in state variable x depends on the level of x D time units earlier. Period length and amplitude are shown for different D values (4.2 to 5.4). Lower left: For two different D values (4.4 and 5.4) the phase plane diagrams (dx/dt versus x) are shown. With increasing temperature the amplitude increases. Thus the path on the limit cycle increases (red limit cycle in lower left diagram), but the process runs also faster: As a consequence, the period does not change much. Lower right: Oscillation of x (y-axis) with time (x-axis) for two extreme values of D (4.4, blue, and 5.4, red curve). For D values less extreme the period lengths are quite close at different temperatures. After [848]*

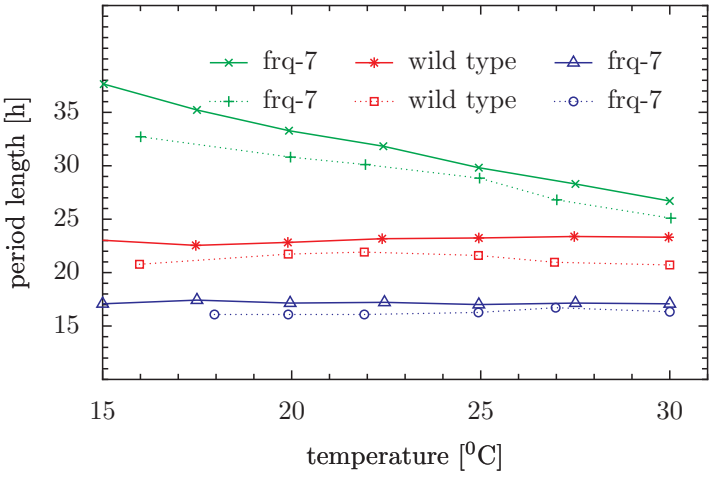

Figure 16.11: *Temperature compensation of* Neurospora *circadian banding rhythm by using the Goodwin oscillator (figure 16.15) model with negative feedback. Temperature dependencies of* Neurospora *mutants frq-7 (green) and frq-1 (blue) are fairly well predicted (circles are predictions, triangles experimentally found) and temperature compensation of wild type holds. After [1282]*

not the clock itself. Slowly growing strains show the rhythm more clearly.

If hyphae are transferred to a new medium, they stay in phase with the mother mycelium. On old medium the rhythm is slowed somewhat. Even if the conidial rhythm does not show up (for instance if the medium is densely seeded), the rhythm is still present, as can be seen by transferring the mycelium to a new medium.

The extracellular pH in the range 4-9 influences the period length of the sporulation rhythm hardly, although the growth rate is influenced strongly by it ([1280]).

CO_2 dampens the rhythm of conidiation ([1310]). This can be seen especially well in the wild type. By aeration the conidia are also produced rhythmically. If the biotin concentration in the medium is low, the wild type shows a clear rhythm of conidiation. Biotin is cofactor of two enzymes, which are involved in dark fixation of CO_2.

Sugars have only a minor influence on the rhythm.

Alcohols, detergents and ionophores such as valinomycin ([455]) influence the circadian rhythm.

For influences of inhibitors see section 16.6.

16.6 How can the mechanism of the circadian clock of *Neurospora* be clarified?

How circadian rhythms function, is not known yet in any of the systems studied including *Neurospora*. As mentioned before, *Neurospora* is however especially well suited to clarify the underlying mechanism.

It is difficult in a complex control system to shut off parts of it in such a way that the whole system is not affected. For-

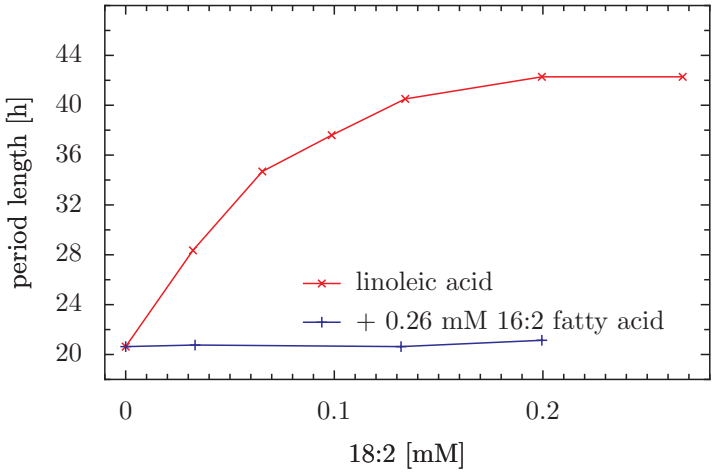

Figure 16.12: *Unsaturated fatty acids such as linoleic acid (concentration at x-axis) lengthen the period of the conidiation rhythm in the mutant cel$^-$ of* Neurospora crassa *until reaching a plateau (red curve). The period lengthening can be reversed by adding a saturated fatty acid (16:0) to the medium (blue curve). After [161]*

tunately the clock mechanism seems to be discrete, since mutants with defects in the amino acid synthesis, certain aspects of lipid synthesis, vitamin synthesis and some other metabolic pathways are *not* essential for the clock. Inhibitors of certain metabolic pathways do not affect the circadian clock. Although the circadian system controls many metabolic parameters, they are well shielded from metabolic variations ([1252]). In the following some results of using different approaches to understand the clock mechanism are presented.

Pharmacological approach Substances or inhibitors are added which interfere with certain parts of the metabolism and it is tested whether the circadian clock is affected by changing its speed and amplitude or by stopping it. Pulses of those substances might phase shift the rhythm. In a number of studies certain substances were administered to *Neurospora* and the effect observed: Nystatin (and valinomycin) pulses phase shift the rhythm. Nystatin influences membranes via membrane steroids such as ergosterol. In this way K^+ is lost and the membranes depolarize.

Unsaturated fatty acids such as oleate, linolenate and linoleate lengthen the period of the conidiation rhythm in the mutant *cel*$^-$. The period lengthening can be reversed by adding a saturated fatty acid (16:0) to the medium (figure 16.12, [161]). The effect depends on the temperature. At 30° it is minimal, at 20° maximal and at 25° intermediate. These fatty acids interfere also with the temperature-compensation.

Saturated fatty acids prolong the period after some days ([161]).

Adding inhibitors is another strategy. Protein synthesis inhibitors such as cycloheximid shift the phase of

the conidial rhythm. Cycloheximid inhibits the 80s protein synthesis. If cycloheximid resistant mutants are treated with cycloheximid, phase is not shifted. Mutants with long periods are cycloheximid insensitive. Temperature-compensation and protein synthesis might be connected with each other. Mutants with a long period might possess a higher protein concentration. Chloramphenicol inhibits the protein synthesis at the 70s ribosomes and shortens the period of the conidiation rhythm ([455]). However, since the l- and d-form are effective, whereas in the protein synthesis only the d-form inhibits, the effect is a different one.

Phosphodiesterase-inhibitors lengthen the period of the circadian conidiation rhythm. This inhibitor increases the cAMP concentration. However, the mutant crisp with low adenylat cyclase concentrations (which metabolizes cAMP) has a normal period length and can be synchronized by a light-dark-cycle. Quinidin, an adenylat cyclase inhibitor, has also no effect.

Genetic approach: Generally, mutants can contribute in various ways to shed light on the mechanism of circadian rhythms. Known biochemical mutants can, for instance, be studied, in order to find out, whether the rhythm was changed. If this is not the case, the corresponding metabolic pathway is not essential for the clock. Furthermore mutants can be used which change clock-properties. One can try to find out the underlying mechanisms which are changed in this particular mutant. They must play a direct or indirect role in the clock-mechanism.

Mutants with defects in biochemical pathways were used to find out whether these pathways are needed for the circadian rhythm. In this way it was for instance shown that the glyoxalate-, tricarbon cycle- and urea cycle are not important for the circadian clock of *Neurospora crassa*.

Resistant mutants were isolated, to study their influence on the circadian rhythm. Experiments with the oligomycin-resistant mutant oli^r have shown that at 22° period is shorter as compared to that of the wild type. This effect is absent between 26° and 30°. Since in this mutant the mitochondrial ATP-synthetase is defect, the ATP-synthesis might be a part of the clock. On the other hand ATP is also important for protein synthesis and ion transport.

Mutants with changed clock-properties were used such as cel$^-$, which lacks temperature compensation below 22°. Palmitate restores the compensation. It was assumed, that membranes might play an important role. However phenethyl alcohol, which changes the phospholipid composition of the membrane strongly, has no effect on the circadian rhythm.

Other clock mutants are listed in table 16.1.

From these and other studies the following conclusions were drawn:

Membranes Membranes play possibly an important role in circadian rhythms. The time delays needed to get oscillations in the circadian range have been explained

by diffusions in membranes. [1096] used membranes in his model for circadian rhythms (see also [1439]). Photoreceptors for the synchronization of circadian rhythms are membrane bound.

It is known that the structure and function of membranes changes in many circumstances in a circadian way. A number of substances which influence circadian rhythms affect also membranes. To them belong fatty acids, nystatin, fusaric acid, steroids, filipin, vanillic acid, ions such as Li^+ and Ca^{2+}, abscissic acid, valinomycin (an ionophor), detergents, hyamin, digitonin, alcohols, morphactines, D_2O. Perhaps mitochondrial effects are of secondary nature and act primarily on membranes. Studies of [1054] are in favor of this interpretation, where he inhibited the plasmalemma ATPases. In this connection mutants are of interest, in which the membranes are changed, such as fatty acid mutants (see also temperature compensation), osmotic mutants, pH sensitive and resistant mutants, ion-sensitive mutants and permeability mutants.

Furthermore, some fatty acids of membranes fluctuate in a circadian pattern (linolenic acid, and 180^0 phase shifted to it linoleic acid). Others do not change (palmitic acid-, stearic acid-, oleic acid) ([1242]). This is found also in the wild type, which does not display a conidiation rhythm. The fluctuations of the fatty acids are therefore not the result of morphological changes in the hyphae during the course of a cycle. If linolenic acid is added in the *cel* mutant to the medium, the period changes considerably (from 21 to 40 hours, see figure 16.12). Fatty acids seem therefore to play an important role in the circadian rhythm of *Neurospora* ([968], reviewed in [850]).

Protein synthesis If protein synthesis is inhibited at certain times by chloramphenicol ([455]), cycloheximid ([1058]), actinomycin, mitomycin or puromycin, phase dependent shifts are observed. However chloramphenicol containing the right- (containing D(+)threonin) as well as the left-turning (containing D(-)threonin) lengthen period. Since only the left turning chloramphenicol inhibits protein synthesis, there is either no connection between the chloramphenicol effect on the period length and the protein synthesis, or the protein, which is responsible for the period shortening, belongs to a special class, which can be inhibited with the left turning chloramphenicol, or chloramphenicol affects the structure or the electron transport of membranes and has no relation to the protein synthesis in mitochondria. In favor of this interpretation is the finding, that valinomycin has the same effect as chloramphenicol.

Role of mitochondria Inhibitors and mutations which influence the energy metabolism and mitochondria change also the circadian behavior of *Neurospora* ([322], [160]). The mutant oli has defective mitochondria due to a changed structure of a mitochondrial protein (ATP-synthetase?). This mutant shows a different period length of the circadian rhythm (18.5 hours). Other mutations which contain higher mitochondrial protein concentrations show also shorter periods.

Calcium, calmodulin, calcium channels, phosphatidyl-inositol-cycle A number of different authors have studied the role of calcium, Calmodulin, calcium channels and the phosphatidyl-inositol-cycle in the circadian conidiation rhythm

of *Neurospora crassa*. The Ca-ionophor A23187 shifts the rhythm in a phase dependent way. Ca seems to be important at the late subjective day for the rhythm ([1055]). In favor of this is also, that calmodulin antagonists administered as a pulse at different phases of the cycle shift the rhythm at these times ([20], [1056]). Ca-channel-inhibitors shift the rhythm at CT5 by 5 hours ([1460]).

[842] has tested, whether the IP-signal pathway is involved in the conidiation rhythm. The reason for it was, that lithium salts lengthened the period of the rhythmic conidiation. Her studies have shown, however, that the IP-signal pathway is not responsible for the lithium salt effects. Likewise the phase shift by blue light does not use this pathway.

16.7 Mutations in the circadian system

From *Neurospora crassa* more than 5000 mutants are known in the meantime. In some of them the rhythmic conidiation is affected (overview [850]). Several of these clock-mutants have lost temperature compensation and in some cases the sensitivity toward light is affected ([923], [922], [476], [320]). In others the period length is changed. Some of these genes are pleiotropic or act in an organism-specific manner and are therefore not informative. The canonical clock-genes are compiled in table 16.1. In the *frq* mutants 9 alleles are known. Further mutants are known, but their influence on the circadian clock is small. Especially the frq mutants were studied intensively. They possess faster or slower circadian clocks as compared to the wild type, but normal growth rates.

The frq gene is on chromosome IVR (see

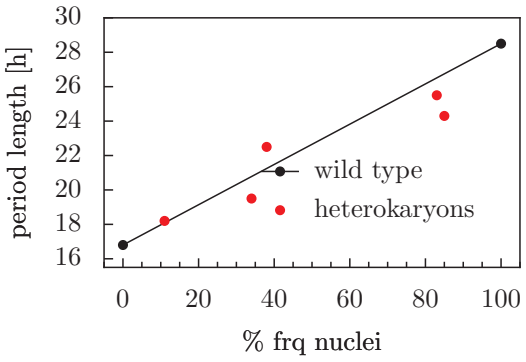

Figure 16.13: *Heterokaryons (see footnote 7) show a gene dosis effect: The phenotypic period length (y-axis) is proportional to the gene dosis of the frq7-nuclei (x-axis). Black dots are the wild type (left) respectively the frq7 (right). After [923]*

figure 16.14). For the rhythm the locus or the gene product play a decisive role, because growth and development of all frq mutants is normal. Usually a mutation lacks one trait and therefore most defect mutants are recessive. However, the frq mutants show incomplete dominance. Heterokaryons[7] show a gene dosis effect: Between the phenotypic period length and the gene dosis of the frq7-nuclei proportionality is found (figure 16.13). All this speaks in favor of a decisive role of frq or the frq-product FRQ for the circadian rhythm.

16.8 The clock mechanism of *Neurospora*

How does the circadian clock of *Neurospora* work, how does it control gene

[7] Heterokaryons are produced by fusing the mycelia of two different genotypes in such a way that different alleles at a locus are in different nuclei, but share a common cytoplasm.

Table 16.1: *Clock-gene mutations which affect the circadian rhythm of* Neurospora. *TC temperature compensation, NC arrhythmicity under some conditions, very long non circadian periods, no synchronization by LD, TS temperature sensitive, NC nutritional compensation. From [921]*

gene name	period length [h]	affected	reference
cel	variable	TC	[969]
chr	23.5	TC	[844]
frq1	16		[425]
frq2	19		[475], [425]
frq3(=4,6)	24	TC	[475], [425], [242]
frq7(=8)	29	TC	[475], [425], [242]
frq9	conditional, NC	frame shift mutation, TC, NC, entr.	[922], [923]
frq10	conditional, NC	frq null, TC, NC	[25]
frq11	conditional, NC	rhythm only below 27^0C	[1057], [474], [905]
prd-1	26	TC	[424], [422]
prd-2	25.5		[420], [424], [475]
prd-3	25	TC	[420], [424], [475]
prd-4	18	TC	[420], [424], [475]
prd-6	short	TS, epistatic to prd-2	[1032]
vvd	23	phase mut., 4 h earlier	[594]
wc-1	conditional-NC		[269]
wc-2	conditional-NC	TC	[242]
pol-1	long		[1112]
rhy-1	conditional		[230]
un-10	long		[923]
un-16	long, conditional	TS	[518]
un-18	long, conditional	TS, RNA polymerase I subunit	[518]

expression and how does it interact with the environment? Several research groups have tried to answer these questions and reviews of genetic ([424], [420], [421], [423]) and molecular biological studies ([337], [27], [919], [905], [343], [845], [921]) are available. A picture of the mechanism which underlies the circadian rhythm of conidiation has emerged, which is complicated. *Neurospora* is a system with circadian control, control by light, metabolic controls and controls by development which all interact.

Here we discuss the circadian control and the control by light and how it might function on the molecular level. First we try to get to know the players, than the play (the interactions between the players), and finally the rules and goal of the play.

16.8.1 The players and the stage

In understanding the circadian oscillations in *Neurospora* and other organisms, it is essential to characterize the important players of the game. Finding a rhythmic variable does not mean it is part of the clock gear. It might just be a hand of the clock. Does it belong to the state variables or only to the parameters which characterize the oscillator? The following criteria should be applied):

- Treatments or mutations which influence phase or period of the circadian rhythm, change also the properties of the components under discussion.
- Disturbances of the components (chemical, mutations) change phase or period of all rhythms observed.
- Increase and decrease of the component influences the observed rhythms, and in an opposite direction.
- In a model which simulates the known properties of the oscillator, the component should be a state variable or a parameter.

In the following some of the essential components of the circadian clockwork are presented. There are certainly more, as suggested by table 16.1.

FRQ: The product FRQ of the frq-mRNA of the frq-gene is one of the main players in the circadian game of *Neurospora crassa*. The frq gene was cloned (by chromosome walk) and sequenced (see figure 16.14 and [26], [27]). It is a 7.7 kb DNA with two transcripts (4 and 4.5kb). The structure is shown in figure 16.16. If one of the two transcripts is deleted, the function is gone.

The mutation frq^3 has affected amino acid 364 (with a period length of 24 hours as compared to the 21.5 of the wild type at 25°C). The mutation frq^7 and frq^8 has affected the amino acid 459 (with a period length of 29 hours). The mutation frq^3 affects the amino acid 364 (with a period length of 16.5 hours). The mutation frq^1 affects the amino acid 482, frq^9 and is a nonsense mutation. frq^2, frq^4, and frq^6 affect the amino acid 895 (with a period length of 19 hours).

All frq-mutations are point mutations in the ORF. They were, with the exception of frq^9, obtained by nitrosoguanin mutagenesis. The null-mutant frq^9 (premature end at 674), was obtained by UV-mutagenesis with a deletion of a base pair, which shifted the reading frame. frq^{10} was obtained by using gene technological methods (without frq locus). In frq^9frq 9 as well as in frq^{10} the period length varies at varying temperatures strongly. Temperature compensation of the rhythm is lost. It is a missense- or a nonsense-mutation in the frq-ORF. Regulation-region, promoter-region

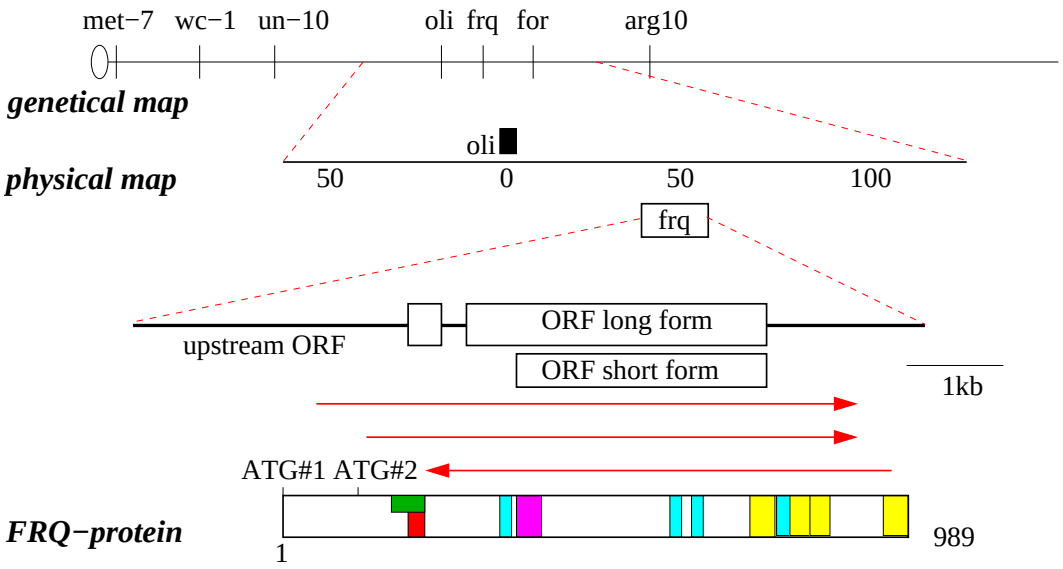

Figure 16.14: *Position and structure of the frq-gene of* Neurospora crassa. *The frq-gene lies on the right arm of the chromosome VII between oli and for. A physical map (below) complements this genetic map and shows the position of the frq gene to be about 50 kb away from oli. The position was determined by inserts of phages (λ) and cosmids (cos). 7.7 kb DNA fragment containing the frq gene shown in center. The frq locus consists among others of a long and a short open reading frame ORF, which codes for a 4.5 kb transcript (top red arrow to right). There might be in addition an overlapping rare transcript (red arrow to left), which could be coded from the counter string. Furthermore an additional small coding transcript is present (second red arrow to right). The large transcript codes for a protein (very bottom) with 989 amino acids. It contains conserved basic domains (blue), TG/SG repeats (violet), a nuclear localization sequence (red), a helix-turn-helix domain (green), conserved acidic domains (yellow) with high serine content, and further acid areas (yellow). After [26] (position of frq) and [77] (frq-locus and FRQ-Protein)*

and the small transcript are not affected. In frq^2 an alanin has mutated to a threonin, in frq^7 a glycin to an aspartic acid. All mutants with longer periods are mutated in the non-conserved region, all mutants with shorter periods (frq^1, frq^2, frq^4, frq^6) in the conserved area.

If the sequences are compared with other *Neurospora*-species, they are highly homologous. The frq-homologue of a distantly related species (*Sordaria fimicula*) is able to turn the frq-null-mutant of *Neurospora crassa* rhythmic again, although *Sordaria* does not form conidia. frq is thus not a gene, which is only involved in the asexual development. Instead, it is a control gene, which is important for the functioning of the circadian clock. FRQ is responsible for the period length *and* the temperature compensation of the *Neurospora* clock. This clock controls different metabolic pathways ([1002], [27]).

WC-1, WC-2 and WCC: Two other players are important, White Color WC-1 and WC-2. They are expressed by the wc-1 and wc-2 genes, both of which have been cloned. wc-mutants have a low frq-expression in darkness and show no circadian rhythm. Temperature is also unable to induce rhythmicity in these mutants. This shows, that WC-1 and WC-2 are components of the clock or closely associated factors of the clock. They are transcription factors containing DNA binding regions, trans-activation domains and 'PAS´ domains which are associated with protein-protein interactions. PAS domains are found in many regulatory proteins with functions in signal transduction and reception of different stimuli (light, chemical compounds, oxygen). Here the PAS domain recognizes binding sites in light regulated promoters and may serve to interact between receptors and signal transduction components. PAS domains are found also in phytochrome and in other clock proteins (for instance in PER of *Drosophila*). This might indicate that clock proteins have evolved from proteins responding to light ([269]). There is furthermore a sequence similarity between WC and PER which apparently extents also to the mouse clock ([19], [774]).

WC-1 and WC-2 dimerize with each other to form the White Color Complex WCC. The PAS domain is used for the dimerization. WCC binds to the promoter of the frq gene at two sites. This activates the expression of the frq gene. The primary transcripts are spliced in a complex way with major effects on the proteins produced. WCC transfers light signals to light responsive and clock-controlled genes (arrows to frq, wc-1 and ccg in figure 16.16). WC-2 is an abundant, constitutive nuclear protein, which provides a scaffold allowing FRQ and WC-1 (which are out of phase) to interact with each other ([309]).

VVD: Recently another player has been found playing its part in the circadian system of *Neurospora.* The vivid gene (vvd) transcribes VVD, a novel member of the PAS proteins. It was cloned and characterized ([594]). Light induces it rapidly, but it is independently controlled by the circadian clock. It is a small protein with a PAS domain. It affects input *and* output of the clock, without being a part of the clock mechanism (vvd null mutants are still rhythmic).

Other players: Other players must be involved in the circadian system of *Neurospora*, since frq-null mutants are still rhythmic (although quite different from

circadian). They are unknown so far, but need to be mentioned here (see subsection 16.8.2).

16.8.2 The play

In *Neurospora crassa* the product FRQ of the frq-gene is an essential component (a state variable) of the circadian oscillator. The mRNA and protein-production of the frq-gene are parts of a feedback system composing the circadian clockwork. The products of the frq-locus regulate themselves ([26], [27]). This seems to be a general theme of circadian oscillators. The principles of it have been simulated by [1283] using a molecular feedback oscillator of Goodwin ([511], figure 16.15).

Mainly from the molecular biological data of the group of Dunlap a more specific model was developed for the circadian clock of *Neurospora*, which is shown in figure 16.16. According to it, the product FRQ of the frq gene is an essential component of the circadian oscillator. The mRNA and the FRQ proteins of the frq gene are parts of a feedback system where FRQ regulates its own expression via the white color complex WCC ([866]). FRQ would thus be a state variable in the circadian system ([27]). That protein synthesis is important during the transduction of the blue light signal to the circadian clock, was known already for some time, since protein synthesis inhibitors interfere with phase shifting by light pulses and phase shift the rhythm in a phase-dependent way if administered as a pulse. FRQ is progressively phosphorylated over time, mainly by a calcium/ calmodulin-dependent phosphokinase. Its level decreases when it is hyperphosphorylated ([1592]).

The frq-gene is read in two forms, sFRQ

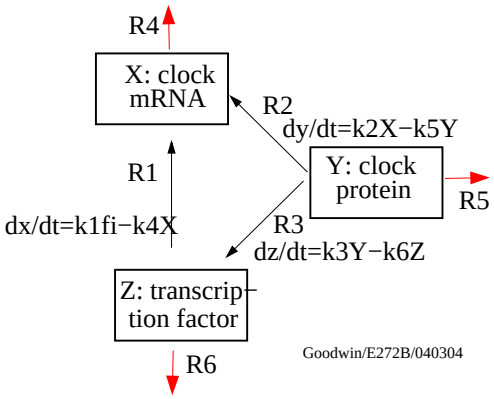

Figure 16.15: *Molecular feedback oscillator of Goodwin. Clock protein Y inhibits its own transcription from the clock mRNA (X) via the transcription factor Z. The production rate of the intermediates Y and Z are linear functions of the concentrations of X respectively Y. X production without inhibition occurs at a constant rate. However, it is inhibited by Z due to the inhibition factor f_i. Each intermediate I (X, Y or Z) is produced and degraded according to $\rightarrow k_s \rightarrow I \rightarrow k_d \rightarrow$I (S), where k_s is the synthesis rate constant (k_1, k_2, k_3) and k_d the degradation rate constant (k_4, k_5, k_6). I in (S) fluctuates between high and low values depending on whether the synthesis reactions are turned on or, due to the inhibition by Z, turned off. As a consequence of (S) the relaxation time (time scale of the approach toward steady state in I) depends on k_d only, and the period length of the oscillation is solely determined by k_d.*
Reaction R1: formation of X, R2: synthesis of Y, R3: production of Z, R4 to R6 (red arrows): degradation reactions. Inhibition factor $f_1 = 1/1 + z^9$. After [1283], based on [511] and [1048]

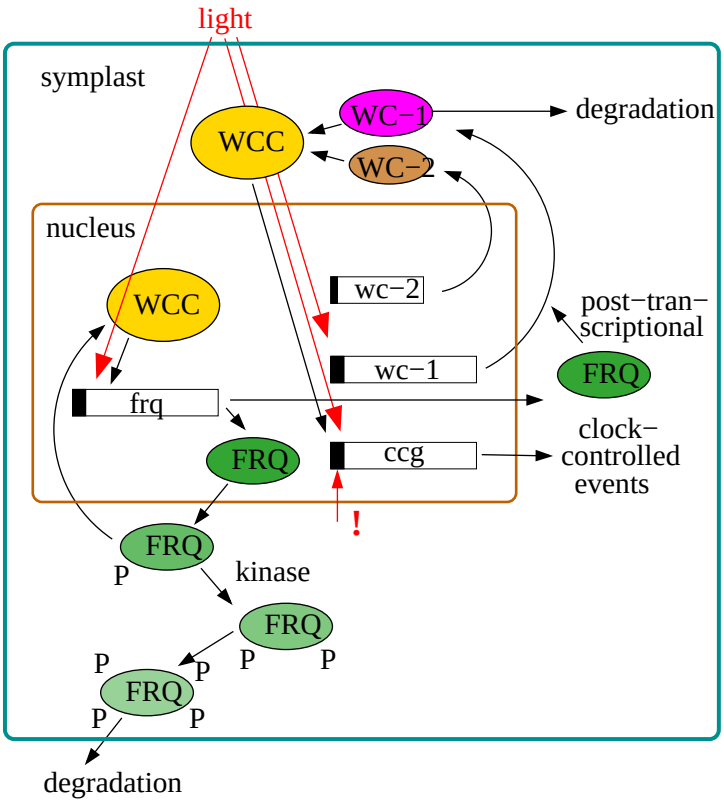

Figure 16.16: *Model of the feedback oscillator of* Neurospora crassa. *mRNA and FRQ protein production of the frq-gene are parts of the feedback system of the circadian clock work. FRQ plays several roles. It regulates the frq-mRNA via trans-acting factors by circadian clock-regulated elements (CCRE's) and leads in this way to the specific transcription at certain times of the day. It furthermore activates directly or indirectly genes, which are in this way controlled by the circadian clock and therefore termed 'clock controlled genes' (ccg's).*
The frq-gene is read in two forms, sFRQ and as lFRQ, which were proposed to be responsible for the temperature compensation (see figure 16.17 and 16.18). By phosphorylation the two FRQ-forms are metabolized.
Furthermore the effect of light as the most important Zeitgeber is shown. Light influences the transcription of the frq-gene, and the protein WC-1 plays a role in the signaling effect of light. In the late night and in the early(vorletztes Bild) morning a light pulse advances the rhythm. This is maximal, if much frq mRNA is present. In the late day and in the early night a light pulse delays the rhythm. Actually at this time FRQ decreases. However, because the light pulse leads to more frq mRNA, it takes longer until the mRNA and FRQ are reduced. Light influences furthermore the clock-controlled genes (ccg's) also directly. WC-2 plays in it a role. It is re-activated in the dark. ([343])

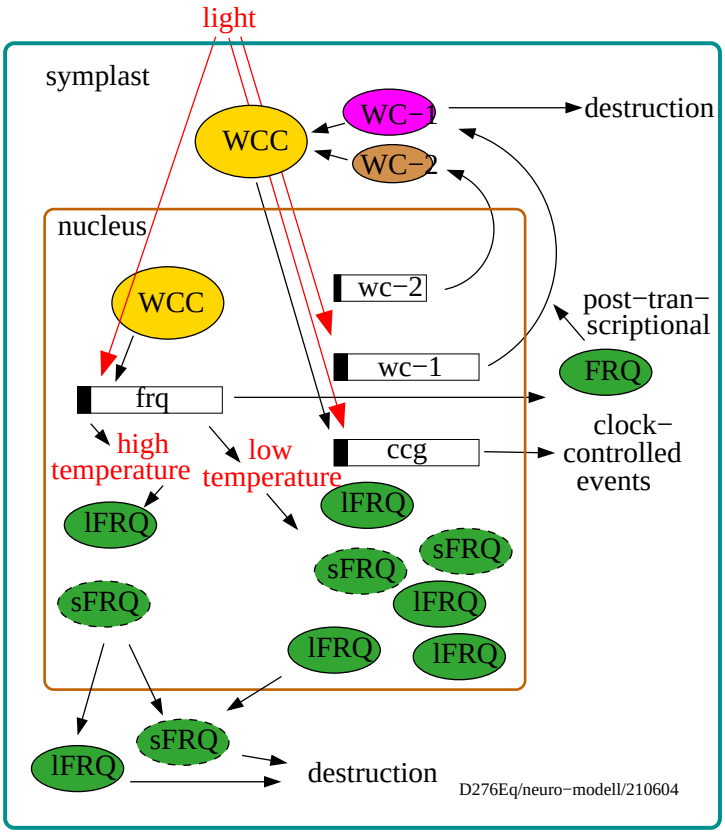

Figure 16.17: *Temperature effects on the frq oscillator of* Neurospora*: The frq-gene is read in two forms, sFRQ (stippled green oval) and as lFRQ (solid green oval), which were proposed to be responsible for the temperature compensation (see also figure 16.18); at lower temperature more lFRQ is made as compared to higher temperature. By phosphorylation the two FRQ-forms are metabolized (very bottom). After [343]*

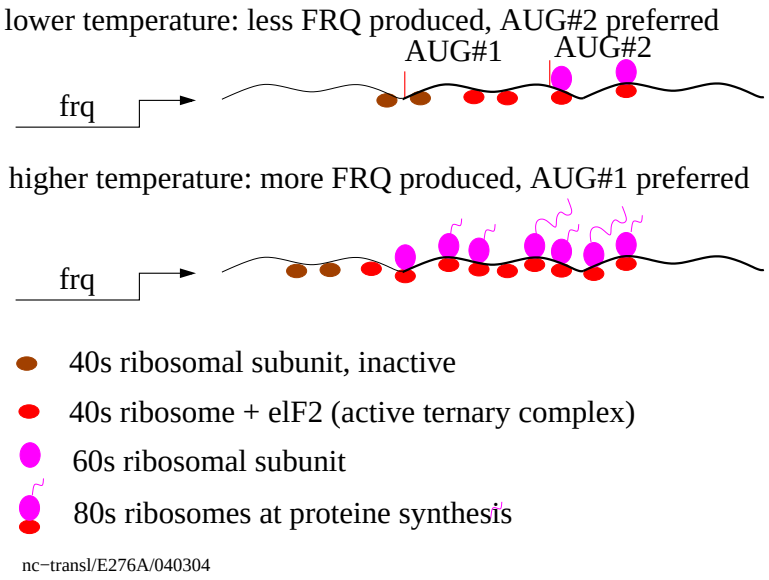

Figure 16.18: *The frq-gene of* Neurospora *is read from the initiation codon AUG#1 or AUG#2, leading to a shorter protein sFRQ or to a longer protein lFRQ, respectively. How much of each is made and thus the ratio of the two forms depends on temperature. In this way temperature compensation of circadian oscillations in* Neurospora *is supposed to arise. By phosphorylation the two FRQ-forms are metabolized. After [343]*

and as lFRQ, which were proposed to be responsible for the **temperature compensation** (see figure 16.17 and 16.18). By phosphorylation the two FRQ-forms are metabolized.

Light affects the circadian system by activating frq expression via the WCC complex. The complex is formed by dimerization of the WC-1 and WC-2 proteins (details on page 387). Figure 16.16 illustrates the pathway light is taking to influence the clockwork, and figure 16.19 shows details of the light effects on the WCC complex and its changes.

It was proposed ([919]) that light affects the circadian clock by switching off the negative feedback of the FRQ on its own synthesis: The repression by FRQ is released (or in spite of the presence of FRQ an induction occurs). This effect of light can be canceled, if protein synthesis or mRNA production is inhibited. Light increases the amount of frq-RNA by a factor of 4 to 25 ([270]). Already two minutes after the light pulse the frq-transcription increases and reaches its highest value 15 minutes later. Afterward it decreases. Transfer to darkness diminishes the amount of frq transcript by turnover. Additional inhibition of the frq-promoter by FRQ resets the clock ([27]). Activation occurs if light is given during late night or early morning and is the first clock-specific event of light. If the synthesis of FRQ is blocked, light does not reset the clockwork anymore ([270]). This model concept explains the effect of single light pulses on the conidiation rhythm in continuous darkness, the behavior under light-dark-cycles and under skeleton

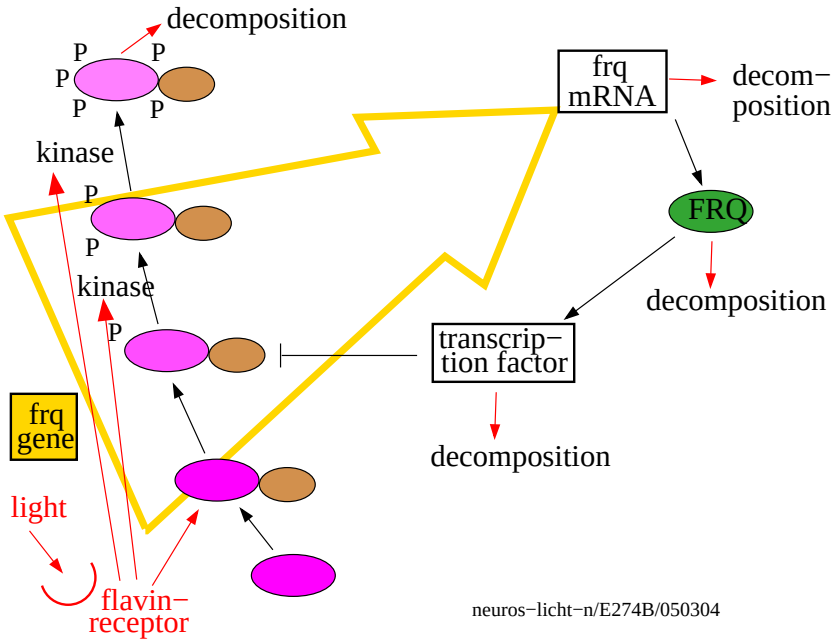

Figure 16.19: *Effects of light on the molecular feedback oscillator of* Neurospora. *Clock protein FRQ (right) inhibits its own transcription from the frq-mRNA (top right) via a transcription factor (below). In light frq-transcription is increased in the following way: Light is perceived by a flavin receptor and interacts (red arrows from flavin receptor) with frq gene expression. The WCC complex (WC-1, lilac and WC-2, brown) plays here the decisive role. WC-1 is constitutively expressed in the dark (bottom). With light it forms the WCC complex with WC-2. This newly synthesized WCC (second from bottom) is inactive both in light and in darkness. A minimal phosphorylated form of WCC (third from bottom) occurs in darkness and acts as a transcription factor for frq (white yellow arrow). It can be inhibited by the transcription factor. Light activates a kinase which increases phosphorylation of WC-1 in the WCC (fourth from bottom). This increases the frq-transcription (large yellow arrow). Furthermore, the transcription factor is no longer able to inhibit transcription. Hyperphosphorylated WC-1 is degraded (top with red arrow). Newly synthesized WC-1 replaces this WC-1 and the WCC becomes inactive again. After [1284]*

photoperiods. It explains also how a light signal delays or advances the rhythm depending on the phase at which it was given (figure 16.16).

The phase shifting effects of light pulses occur, according to this model, in the following way: Light, received by the photoreceptor, quickly induces frq expression, if given during late night or early morning (the circadian activation would take several hours). The amounts of frq mRNA and FRQ increase earlier as without light pulse and advance the rhythm. Light in the late day and early evening again immediately increases the expression of frq and FRQ synthesis. This postpones the FRQ turnover and the rhythm is delayed. There is a close correlation between the dose response of frq mRNA and phase shifting in response to the fluency rate. If the synthesis of FRQ is blocked, light does not reset the clockwork anymore ([270]).

WCC transfers light signals also to light responsive and clock-controlled genes (arrows to frq, wc-1 and ccgs in figure 16.17, [28]) independent of its effect on the clock. Other genes are known which are controlled by both the clock and directly by light. An example is shown in figure 16.20. It is the eas-promoter and the position of the regulatory elements is shown, among those responsible to light.

Finally, the vivid gene (vvd) is involved in the play. It affects input and output of the clock. It is induced by light, but independently controlled by the circadian clock without being a part of the clock mechanism (see figure 16.21).

Temperature

Like light pulses, temperature pulses are able to phase shift the circadian rhythm of *Neurospora* ([452], [906], [510]). Within certain limits period length is not much affected by the environmental temperature.

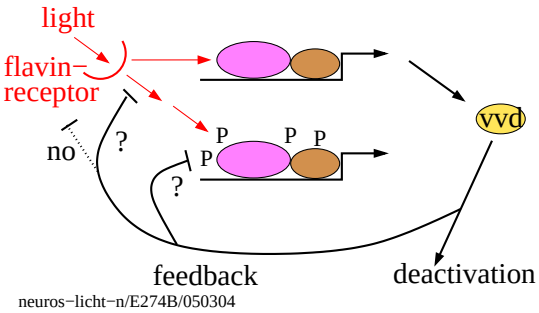

Figure 16.21: *The vivid gene vvd is induced by light received by a photoreceptor PR via the transcription factor WCC (consisting of WC-1, violet, and WC-2, brown, top). It feeds back to either (the lower ?) the input pathway of light to the transcription factor WCC of the frq gene (bottom) or to the light signal transduction pathway from the photoreceptor to the transcription factor WCC of the frq gene (the higher ?), but not (no) to the photoreceptor (dashed pathway). The vivid gene is thus induced by light, but independently controlled by the circadian clock without being a part of the clock mechanism. After [901]*

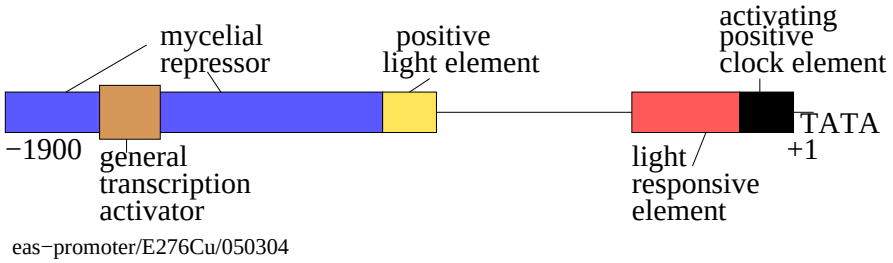

Figure 16.20: *Position of regulatory elements of the eas-promoter of* Neurospora. *It is the promoter of a clock-controlled gene (ccg) with 1900 base pairs, a mycelium-repressor (blue), a general transkription-activator (brow), a positive light-element, an additional element which reacts to light and an activating positive clock-element. CAAT and TATA-box are indicated. After [343]*

Both, temperature pulse effects and temperature compensation of the clock must be explained by a reasonable model based on molecular events.

Temperature effects in *Neurospora* are mediated by post-transcriptional control ([906]). There are two different FRQ forms from two initiation codons made during translation, namely sFRQ and lFRQ. Either form would be sufficient for a functional oscillation at some temperature, but for a robust overt rhythm both are necessary ([905]). Not only the amount of FRQ depends on temperature, but temperature determines also the ratio of sFRQ to lFRQ by favoring different initiation codons at different temperatures. If one of the initiation codons is deleted, the temperature range permitting rhythmicity is reduced. This adaptation mechanism thus extends the physiological temperature range for clock function ([474]).

Resetting the rhythm by a temperature step reflects also post-transcriptional regulations. Although the oscillations at different ambient temperatures are alike, the mean level around which the FRQ values oscillate are different. At a higher temperature this mean level is higher (illustrated in figure 16.22).

A temperature step up or step down corresponds to a different phase of the clock, although the components are not synthesized or turned over. After the step the relative levels of frq and FRQ are assessed according to the new temperature, and the feedback loop responds rapidly and proportional to it in its dynamics. If there was not enough FRQ after the temperature step to shut down WCC, more is made. In this way unlike a light pulse temperature resets the rhythm immediately and from within the loop. Even small temperature changes can affect phase stronger than light pulses.

Temperature compensation is supposed to be the result of expressing the two different kinds of FRQ in different amounts at higher and lower environmental temperatures ([905]). At higher temperatures more sFRQ is made, at lower temperatures more lFRQ. Thus the ratio of the two FRQ's to each other depends on the temperature. In this way temperature compensation of the circadian oscillations in *Neurospora* is supposed to be established (figure 16.16).

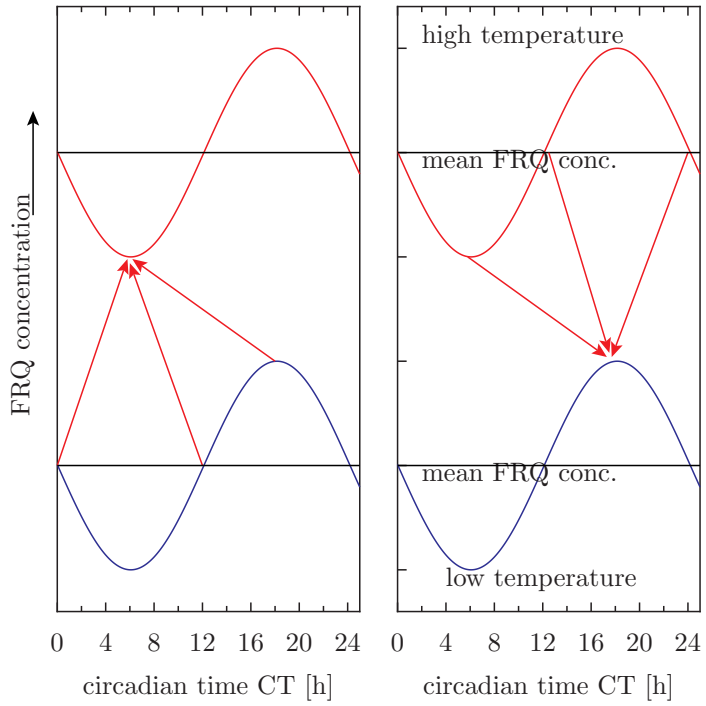

Figure 16.22: *Resetting the clock of* Neurospora *with a temperature step-up (left) resets (red arrows) the rhythm to dawn, where the level of FRQ (in the upper curve) is low, with temperature step-downs (right) to dusk, where the FRQ level is high, independent of the phase of the cycle at which the step occurs (origins of the corresponding red arrows). After [339]*

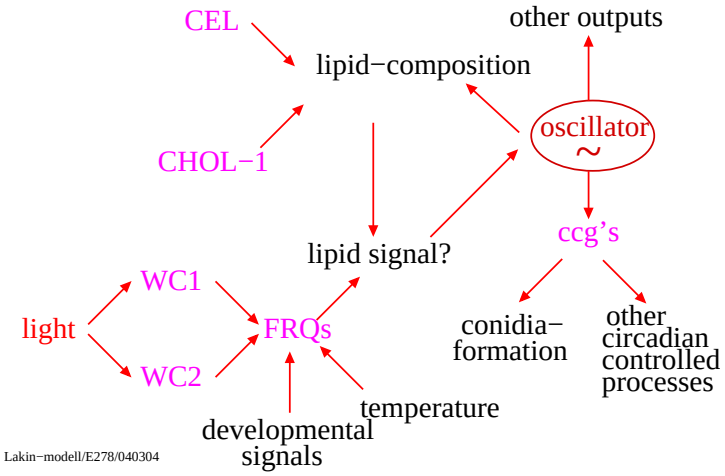

Figure 16.23: *According to Lakin-Thomas FRQ is not a direct part of the circadian oscillator, but rather a part before the oscillator. Light acts via WC-1 and WC-2 on FRQ (temperature and developmental signals affect also FRQ). FRQ influences the circadian oscillator via lipid-signals. Experiments with the mutants cel and chol-1 speak in favor of it. CEL and CHOL-1 affect the lipid composition and thus the lipid signal and the oscillator. The circadian oscillator controls 'clock controlled genes' (ccg's), which influence conidiation and other processes. The circadian oscillator possesses also other outputs and feeds back onto the lipid composition. After [845]*

FLO oscillator The role of FRQ has recently been re-interpreted in two directions: One group questions, whether it is indeed an essential part of the circadian clock work (a gear). They claim, that FRQ affects only processes before the actual oscillator which (via lipid-signals?) act on the proper oscillator ([1251], [846]). It was proposed ([1251]), to place transcription and feedback of the protein on its mRNA-formation outside the actual oscillator (figure 16.23).

The other group adds another oscillator (or perhaps several additional oscillators?) to the FRQ-oscillator (so called FRQ-less oscillator FLO). Although the FRQ oscillator is required for circadian rhythmicity, it is probably not sufficient ([681]).

The reasons for assuming an additional oscillator are initial reports of the frq9 mutant ([922], [923]). They showed that this strain still expresses rhythmicity, albeit one lacking several characteristics of proper circadian rhythms. Thus the rhythm appears only in a fraction of the race tube cultures, the period length is quite variable (12 to 35 hours), the rhythm can not be entrained by light cycles, and it lacks temperature compensation and nutritional compensation. It is reminding us of the strange rhythm found in *Thalassomyxa australis* (see section 18.4) and might belong to a developmental rhythm.

16.8.3 The goals of the play

The goals of the play are the following:

Provision of a reliable clock: The mechanism of a circadian oscillator consisting of interlocked positive and neg-

ative acting feedback loops does not only determine the period length of the circadian clock, but provides also robustness and reliability to it. The level of FRQ oscillation and with it the robustness of the overt rhythm increase with the amount of WC-1 and WC-2 ([1592]).

Synchronization by light: A circadian clock is able to run and control clock dependent events even under constant conditions of light (or darkness) and temperature. However, in nature the clock has to be synchronized to the 24 hour day. Otherwise it would quickly run out of phase with the day/night cycle and could not serve as a reliable clock anymore. We have seen, that photoreceptors and transduction pathways to the clock serve this purpose.

Synchronization by temperature: Temperature cycles are in *Neurospora* even stronger time cues as light/dark cycles are. This might be important for a fungus which often growth on substrates which are not exposed to the daylight.

Temperature compensation: In addition, the circadian clock mechanisms of *Neurospora* provides for temperature compensation which is important for a reliable clock.

Photoperiodism in *Neurospora*? It has been discussed recently whether the seasonality of spore discharge which is often found in fungi applies also to *Neurospora* and whether it is photoperiodically controlled ([1253]).

16.9 Output pathways and control of overt rhythms

We have seen, that the circadian system of *Neurospora* and probably of other organisms are more complicated than originally anticipated. The last sections dealt with the clockwork and the way how to study it. Since the clock work is so far not well known, we have to use the hands of the clock to infer on clock properties or use mutants which affect it. However, the output pathways of the clock and the way overt rhythms are brought about by the clock are in itself important parts of the circadian system and worth to be studied. Furthermore, by following the pathways from the overt rhythms back to the clock driving them should finally help to understand the clock mechanism.

The best studied circadian rhythm in *Neurospora* is the switch from surface- and sub-surface growth to aerial growth of the hyphae and the subsequent conidiation. The rhythmic conidiation is shown only at the growth front of the mycelium, while growing on solid medium. There it is decided whether aerial hyphae, conidiospores and carotenoids are formed or not.

Many biochemical rhythms are connected with this developmental switch. Examples are the mass of the hyphae, aerial hyphae, hyphal ramifications, formation of a septum, discharge of mature conidia, division of the nucleus, glycolysis, lipid metabolism, the glyoxalate cycle, the tricarbon cycle, the deposition of lipids, the formation of carbohydrates, CO_2 production, activity of a number of enzymes. It has, of course, to be shown whether these examples are caused by conidiation and therefore rhythmic. Alter-

natively, they could occur independently from conidiation. In order to show, for instance, that certain enzymes show a circadian rhythm independent of the conidiation rhythm, the morphological changes associated with it must be prevented. This can be achieved for instance by using liquid cultures (see page 359). Not only from studies in *Neurospora*, but also from other organisms it was found that the circadian clock affects mainly enzymes at crucial points of metabolism, which seems to be a general principle of circadian control.

There are other events in the life cycle of *Neurospora* which are under circadian control. The energy charge (see page 359) fluctuates circadian ([304], [1339]), the heat shock proteins do ([735]), and ascospore discharge is also under circadian control (Brody, unpublished). The period length corresponds to that of conidiation.

The mRNA and rRNA as a whole do *not* fluctuate in a circadian way. However, many genes are clock controlled ([920]).

16.9.1 Clock controlled genes

Genes which are expressed rhythmically even under constant conditions with a period length reflecting the strain genotype are called clock-controlled genes ('ccgs´). Loss of function in these genes does not affect the clock. They are restricted to the clock output and have to be distinguished from genes which are regulated by development and from those which react to environmental changes. Clock controlled genes are driven by the circadian clock by factors which transmit phase specific time informations to their target genes (see figure 16.24). The details of such control of overt rhythms have been summarized by [921]. Quite a number of ccgs are known in the meantime and many will be found soon by using differential screens and microarray analysis. Their functions are mostly known (table 2 in [921]). The ccg-2 for instance is identical with the *eas* gene and codes for hydrophobin. It contains a 68 basepair promoter sequence which is necessary and sufficient for the rhythmic expression. There are protein factors that bind specifically to this sequence at certain phases of the day. In this way the gene can be coupled to and uncoupled from the clock ([79]).

How time is read from the clock by the cell is not well understood. As we have seen before, transcriptional and translational steps are involved. An efficient way to study it is for instance by subtractive hybridization of morning versus evening mRNA using time of day specific cDNA libraries ([80] and the *Neurospora* cDNA sequencing project http://www.genome.ou.edu/fungal.html).

The next step would be to characterize the promoters of ccgs. These clock control regulatory elements (CCRE´s) would define clock boxes. Trans-acting factors that bind and control CCRE´s have to be isolated. By moving backward this cascade, one would finally isolate factors which interface with components of the clock mechanism. Some of these steps might be specific for different organisms, others might be conserved and generally found in many organisms.

ccgs are often additionally regulated by light and by developmental steps (see table 2 in [921]). That implies in addition to the clock controlled regions specific regions that confer development and light regulation to the gene expression (for instance in *eas*).

The strict differentiation between ccgs and clock genes has recently been blurred. For instance, expression from the frq gene

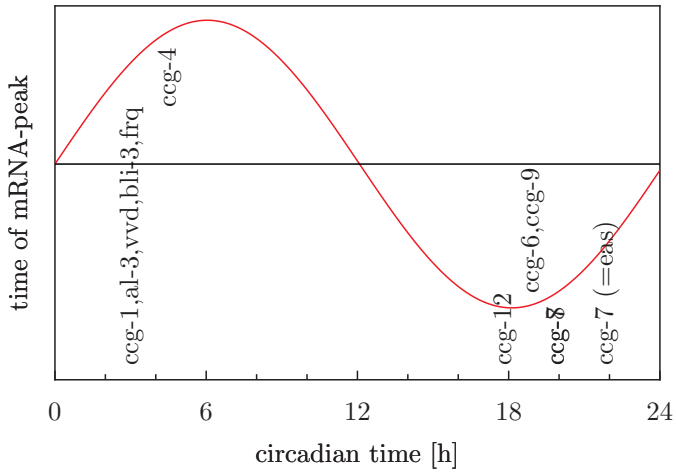

Figure 16.24: *Peak time of mRNA in clock-controlled genes of* Neurospora. *During subjective late night and early morning: ccg-1 (identity unknown), al-3 geranylgeranyl pyrophosphate synthase, vvd (vivid, light repressor), bli-3 (unknown identity), frq (clock component, transcriptional corepressor) and ccg-4 (unknown identity). During subjective evening and early night: ccg-12 (or cmt, copper metallothionein, ccg-6 (unknown identity) and ccg-9 (trehalose synthase), ccg-8 (unknown identity), ccg-7 (glyceraldehyde 3-phosphate dehydrogenase), ccg-2 or eas, hydrophobin). From table 2 in [921]*

is also under clock control and in addition
directly controlled by light.

17 Rhythmic deposition of layers

Corals tell us, that 400 million years ago a year consisted of 400 days; cockroaches add daily a new chitin layer to their skeleton thus increasing its strength.

17.1 Coral clocks

Annual rhythms in organisms might be the result of 'proximate' factors controlling, inducing or interrupting directly developmental processes. An example was given already under the chapter 'Annual rhythms in plants'.

The deposition of calcium-carbonate in corals is a further example. Corals are inhabitants of tropical oceans. They belong to the phylum *Cnidaria* and to the class of *Anthozoa*. Hard corals secret a foot-like support ('epithek´) below the animal. It consists of layers made of $CaCO_3$. Each night a new layer is secreted (figure 17.1). There are about 20 to 30 layers per millimeter, and they can be measured with a micro densitometer. The tides modulate the deposited layers and there are also annual fluctuations to be seen. They are caused by the temperature changes of the sea water during the summer and winter months. If the number of daily depositions are determined per year, they amount to 365 in living corals, reflecting faithfully the number of days in a year.

Fossil corals show also these layers (figure 17.3). Determining the number of layers per year in suited fossil corals leads to an astonishing result: Corals from the Devon 400 million years ago show 400 lay-

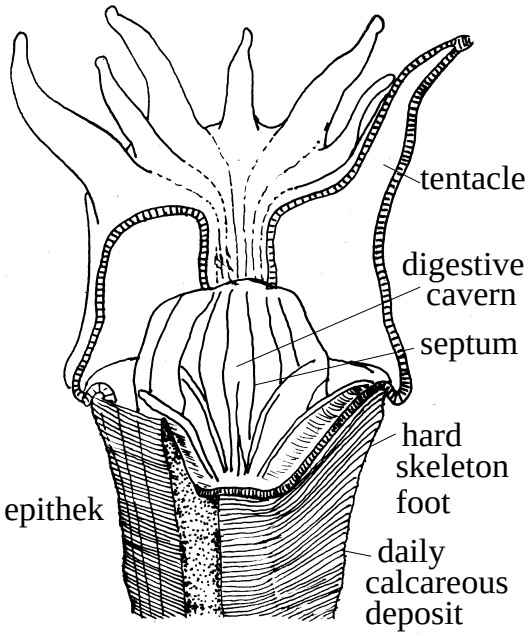

Figure 17.1: *Coral with hard skeleton foot, consisting of daily layers of calcium carbonate ($CaCO_3$). The surface of the foot is called epithek. On top of this epithek the coral with tentacles, gullet and a digestive cavern with septum. After [1279]*

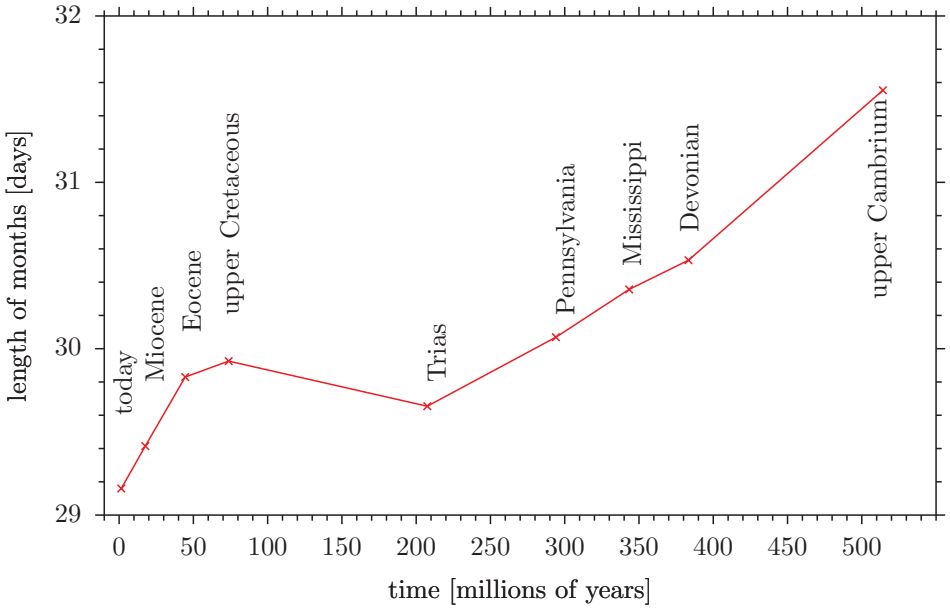

Figure 17.2: *Number of $CaCO_3$-layers deposited during the course of a year (left y-axis) in the foot (epithek) of fossil corals from various periods of the earth history (upper x-axis: Age of the era of the earth. Lower x-axis: names of the era). The right y-axis shows the length of the day in the corresponding eras. After Johnson and Nudde in [1263]*

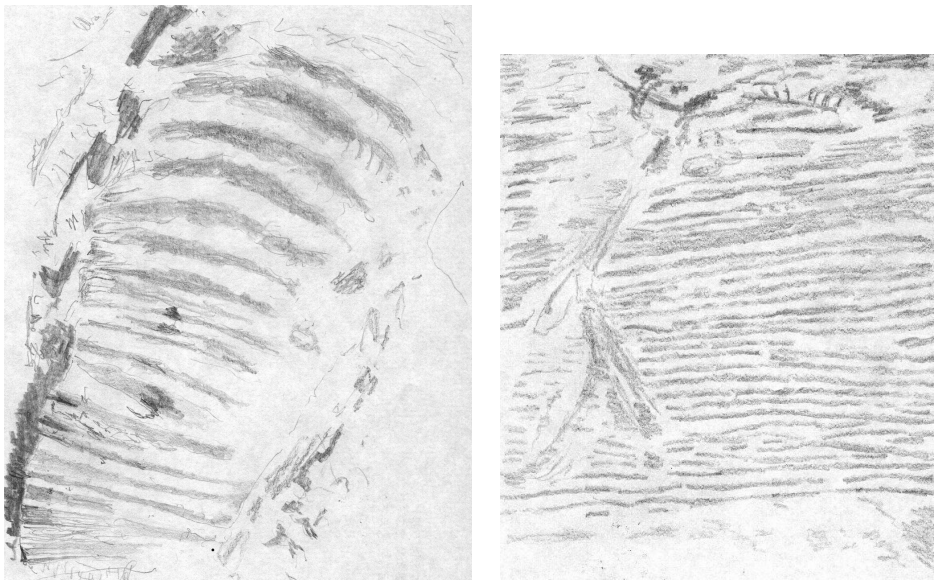

Figure 17.3: *Annual (left) and daily (right) layer formation in the foot ('epitheca') of a fossil coral. From [1279]*

ers per year ([1133]). This would mean, the Devonian year consisted of 400 instead of 365 days. It is indeed a known fact that the moons distance to the earth is slowly increasing because of the tidal frictions. In the same way, the turns of a skater slow down if she extends her arms, the revolutions of the earth-moon-system will become slower if the moons distance increases. Calculations of observed measurements showed, that a day in 10 000 years increases its length by 2 seconds. Thus, a day 400 million years ago was only 22 hours long. Since the path of the earth around the sun has not changed, a Devonian year consisted indeed of 400 days. This is the reason for finding in the epithek of fossil corals more layers per year as in corals of today (figure 17.2).[1] Tidal rhythms and monthly rhythms are also recognizable in fossils. If the 'casts' of fossil corals from the middle Devon are compared with the physiological processes of current corals leading to the structural patterns in the epithek, 13 instead of 12 months per year are found. A month nowadays is thus longer as it used to be 400 million years ago.

Such geo-chronometers were described already by [1558] and intensively studied ([1549]). There is an interesting article ([1279]) and a book ([1263]) on this topic. Shells, cephalopods and stromatolithae (algae, *Conophyton*) show also these depositions. Recently periodic structures (annual: [273], daily: [1235]) were found in bones of dinosaurs living some 150 million years ago.

[1] The lunar tidal friction slows the rotation of the earth by 18.1 seconds per one million years, the tidal sun-earth-interaction by 5 seconds. This adds up to 23 seconds per one million years [709]

17.2 Rhythmic depositions in the cuticle

Immediately after a molt of an insect the cuticle is not yet pigmented, soft and thin. After having extended to its final size, it becomes pigmented and hardens in the course of hours. Thickening of the cuticle might, however, take many more days. The epidermal cells of the endocuticle secret the cuticle often not uniformly distributed over the day, but in a rhythmic way at certain times of the day. During the night chitin is deposited in specially organized lamellae as crystallites. During the day chitin is secreted to the same amount, but not in lamellae. In this way two layers are produced per day, which differ if looked at under the polarization microscope: a double braking (lamellated) and a dark (non-lamellated) growth layer (figure 17.4, [1081]). In locusts the cuticle is rhythmically deposited under continuous darkness for more than 2 weeks. The Q_{10} is 1.04 (for temperatures between 22 and 30^0C) and the period is 23 hours long. In continuous light of 100 lux the rhythm damps out in a day. The chitin lamellogenesis is uncoupled from the clock under these conditions. Synchronizing light is not perceived via normal photoreceptors and the neuroendocrine system: A darkened leg continues to deposit chitin rhythmically under continuous light. The epidermis cells are directly light sensitive. The threshold of sensitivity lies between 1 and 10 lux, the most effective wavelength are between 435 and 520 nm. Rhythmic chitin deposition was found also in *Dolichopoda hinderi* in continuous darkness at 13^0C ([1080]). At higher temperatures the layers were thicker, but the final thickness was the same. At higher tempera-

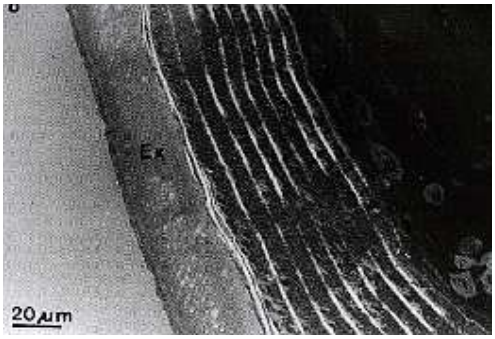

Figure 17.4: *Daily secretion of the epidermal cells of the endocuticle of cockroaches (*Leucophaea maderae*). During the night chitin is laid down in differently organized lamellae as compared to the day layers. Two layers are formed per day, which look different under the polarizing microscope: a double braking (lamellated) and a dark (non-lamellated) growth layer. After [1560]*

tures less layers were thus formed.

A further example is the New Zealand weta *Hemideina thoracica* (Orthoptera: Stenopelmatidae). Layer formation is controlled by an oscillator, which is not identical with the oscillator controlling locomotor activity ([1523]).

Daily growth layers were also found in the inner muscle attachments (‘apodemes´) of flies and mosquitoes. They can be used to determine the age of field-caught *Drosophila* flies ([722]).

18 Significance, evolution and selective advantage of circadian rhythms

This chapter consists mainly of speculations. Although rhythms are widespread and a property of complicated systems, its selective advantage has been shown in a few cases only so far. The rhythmic change in shape of Thalassomyxa australis *might serve as an example of an ancient circadian clock type without temperature compensation and unusual synchronizing behavior. The mechanisms underlying circadian rhythms seem to use feedback coupling of gene products upon the own transcription, but the tooth wheels of the clock work can be quite divergent. At the present state one can speculate only how circadian rhythms might have evolved. As long as the mechanisms of circadian clocks are unknown we are not able to clarify the question, whether these clocks developed in a convergent way in the different kingdoms of organisms.*

18.1 Oscillations are found in all complicated systems

All complicated dynamical structures tend to oscillate. This is true for machines, the economy, biochemical networks, physiological processes, ecosystems, to name a few. In organisms with their complicated metabolism, numerous control circuits and feedbacks rhythms are common and found from prokaryotes to man.

Rhythms are not only unavoidable, but they can be also of advantage. Engineers in the chemical industry have tried to improve process controls by deviating rhythmically from average conditions. Organisms use this strategy often. For instance the gained energy during glycolysis-oscillations of yeast is increased ([1234]). In order to survive during food deprivation the social amoeba *Dictyostelium discoideum* discharges cAMP as pulses instead of randomly distributed ([1060]).

18.2 Why circadian rhythms are not precisely 24 hours long

Why is the period length of circadian rhythms not exactly 24 hours, but as a rule shorter or longer? There must be one or several advantages which led during evolution to these 'free running ' clocks.

One advantage is a better synchronization. If the period length of the circadian clock would be quite close to 24 hours, it might take several days, until the difference is noted by the system and countermeasures taken. It is therefore a better strategy to use a clock the period of which is not exactly 24 hours and to reset it each day anew. In this way the phase relationship to the day-night-cycle becomes more stable. A clock, the period of which deviates from the 24 hour measure of the environment, has a further advantage: The phase relationship of the internal oscillator to the environmental rhythm depends

on its period length ([407]). If, for instance, an animal for some reason wants to wake up early ('early bird catches the worm'), the period of the circadian clock should be short. A long period, on the other hand, would make the animal wake up late and go to rest late.

18.3 Selective advantages of circadian rhythms

To produce circadian rhythms, during evolution out of the already existing rhythms only those had to be selected, the period length of which was in the range of 24 hours. Circadian rhythms are wide spread among organisms and possess a special adaptive value. An internal clock can be used in three ways: It can continuously be asked for the time, it can be used as a stop watch in order to determine the length of a time period, and it can be used as a timing program, which allows to run at certain times certain activities.

We got to know already in the preceding section some examples. A circadian system allows an organism to prepare for the 24-hour time structure of the environment, such as the regular changes in the light-dark-cycle and in the temperature fluctuations of the days. If a daily clock is present, animals are able to calculate the course of the sun and use this in addition with the angle of the sun to some food source or position of the home place to exert sun compass orientation (page 177). Photoreceptors can change their sensitivity toward light with the course of the day and adapt to the various light intensities. Processes which are not compatible with each other such as photosynthesis and nitrogen fixation in *Cyanobacteria* (page 131) or phototaxis and chemotaxis in *Chlamydomonas* ([191]). Circadian rhythms time in this way the interconnections of biochemical events and the energy metabolism. Enzymes can exist in day- and night-forms such as the catalase in *Arabidopsis thaliana* ([1610]) and the PEP-carboxylase in CAM, which occurs in a daily rhythm (page 153). Circadian rhythms are the basis of photoperiodic timing, which take care that growth, development, dormancy and other events occur during the appropriate time of the year (section 13.1 and following sections).

Rhythms can be used to set the time at which special events ought to occur. The eclosion of *Drosophila pseudoobscura* out of its puparium is such an example. It was already presented on page 307. A more complicated control of eclosion is found in the marine midge *Clunio marinus* (see page 192). Here two timing systems coordinate eclosion to occur during the correct time of day and at low tides. One system is circadian, the other occurs every fourteen days ([1075]). The chances of fertilization of the Palolo worm are increased enormously by discharging the gonadal products at certain times of the day only (in the morning) and in a special phase relationship to the moon cycle (first day after the last quarter of the moon) in two month of the years (end of October, begin of November) (section 11.21).

In other cases the selective advantage is unknown or disputed. Leaf movement rhythms were described already 2300 years ago and were probably known to man much earlier. The leaf movements of *Tamarindus indica* were put to paper by Androsthenes 400 before Christ on the march of Alexander the Great to India ([154]). Biologists such as Darwin, Pfeffer, Sachs, Bünning tried to understand the selective advantage of these movements (see

page 236 and following pages). The significance is still disputed even today; perhaps several advantages are connected with it ([187], [747], [399]).

Photoperiodic time measurement is done according to [180] by a circadian clock. Especially in organisms on the land many events are photoperiodically controlled. Probably photoperiodic mechanisms developed in the plant and animal kingdom independent of each other and different principles were used ([186]).

If circadian rhythms are of advantage to organisms, disturbances of the circadian system or abnormal conditions should lead to problems. This hypothesis was tested experimentally. The daily rhythm of flies was shifted by using a changed light-dark-cycle. This treatment simulated traveling through time zones from east to west or *vice versa*. 90% of the flies which were kept under a 12:12 hour light-dark-cycle without phase shifts had died after 125 days. Of the phase shifted one 90% were dead already after 98 days ([34]). This indicates that phase shifting shortens life expectation of these flies (section 14.18).

Phase shifting of the light-dark-cycle every third day shortened the life time of mice, tumors grew to a larger extend and the immune system was suppressed. If these animals were treated with melatonin the unfavorable influence was absent ([893]).

A similar experiment was done by [1164] using *Drosophila melanogaster*. Control flies were kept in 12:12 hour light-dark-cycles, other groups in shorter (10.5:10.5 hours) or longer (13.5:13.5hours) light-dark-cycles or under continuous light. Flies kept in a 24 hour day lived significantly longer as compared to the other groups (section 14.17).[1] These and other experiments in plants ([1554], [635], [1554], [637]) show, that it is better for organisms if their circadian oscillators are driven by the natural 24-hours-period.

In *Cyanobacteria* selection experiments were done by [703] . They used a mixture of wild type and mutants with a changed period length and kept the cultures under artificial 22- and 30-hour-days. An arrhythmic mutant was also tested. The algae, the period length of which was closest to the offered cycle, replaced the competitor. The wild type replaced the arrhythmic mutant (figure 18.1).

The hypothesis, that circadian rhythms are of advantage to organisms, was tested also in another way. For this purpose a special light pulse was given while flies were eclosing from their pupal case. This light pulse renders the eclosion rhythm of *Drosophila pseudoobscura* flies according to a method of [1569] arrhythmic. It was expected, that the flies are less successful in eclosing in the arrhythmic group or that they lived shorter after eclosion. No difference was found, however ([944]). To conclude, that animals without an intact circadian clock are as successful as the control is, however, premature. It could namely be shown, that eclosion is controlled by another circadian clock as is locomotor activity. The treatment had stopped the eclosion clock, but not the clock controlling the locomotion of the animals in a circadian way.

Therefore another experiment was conducted using house flies *Musca domestica* (section 14.19). The duration of life was determined. If the animals are kept under

[1] The results could, however, in more recent experiments not be reproduced even by the same authors (personal communication)

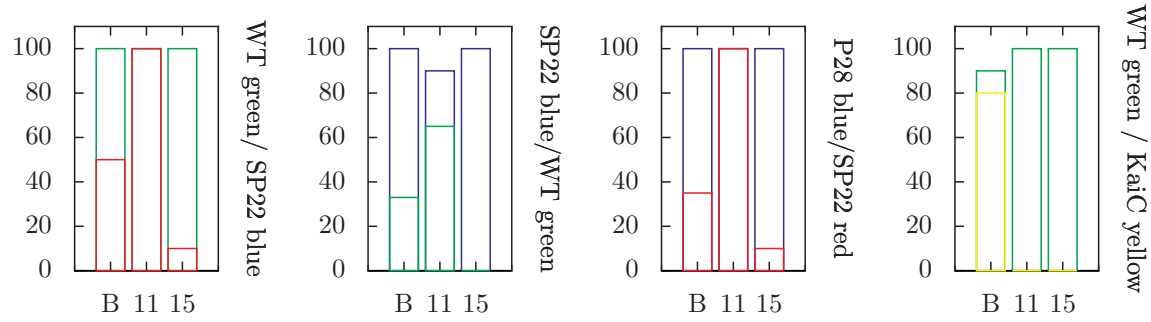

Figure 18.1: *Selection experiments of [705]. Top row: Mixtures of wild type (free run period 24 hours) and the period-mutant SP22 (free run -period 22 hours) were kept under 22- (11 hours light, 11 hours darkness) and 30-hours-days (15 hours light, 15 hours darkness). After 27 days the wild type was more numerous under the 30-hour-day as compared to the mutant. In the 22-hour-day, however, the mutant had completely replaced the wild type. Second row: The mutant P28 (free run period 28 hours) had replaced under a 30-hours-day after 27 days the wild type completely. Under a 22-hour-day, however they were replaced by the wild type. In the third row P28- and SP22-mutants were mixed with each other. In the 22-hour-cycle SP22 pushes away the mutant P28, in the 30-hour-day it was the other way round. Fourth row: The arrhythmic mutant KaiC was replaced by the wild type both in the 22-hour- and the 30-hour-day. On the y-axis the ratio of the mixed cultures are plotted.*

continuous light of 0.01 lux, most of the animals showed first a circadian rhythm of locomotion. But with time this rhythm is lost, the animals become arrhythmic. However, at the end of the experiment from day 75 to 85, when most of the flies had died already, 50% of the survivors where rhythmic, much more as in the days before. This might indicate, that *those* animals live longer, the circadian rhythm of which was intact under the continuous light conditions until the very end of their life (Engelmann, unpublished).

There are furthermore indications that in endogenous depression of man the circadian rhythm is abnormal ([549]).

It is unknown how circadian rhythms have evolved. Different scenarios can be imagined:

1. [776] has speculated that originally it was not the synchronization with the rhythmic structure of the environment, why rhythms arose. Instead it was necessary to coordinate the metabolism between the endosymbiontic precursors of the organelles and the archebacteria-like ancestors of the eukaryotic cytoplasm. Without this coordination chaotic oscillations would occur between the autonomous compartments. Later the circadian rhythms would be selected. They had the further selective advantage to couple the energy metabolism to the daily rhythm.

2. [1572], [1575] proposed that 'dawn warning' might have led to circadian rhythms. The organisms were now able to anticipate the onset of the noxious sun light before the arrival of

the sun, because the daily clock announced it in advance.

3. The same author points out, that rhythms have advantages for the cell in comparison to events running under equilibrium conditions. Likewise chemists improve process controls by rhythmic deviations from the average values. Compartmentation in space and time allow incompatible reactions to occur. Much later these rhythms have adapted to the rhythms of the environment by selective pressure.

4. A further proposal by Winfree: The daily rhythm of cells was imprinted upon the cells by the light-dark-cycle and/or the temperature change. Later the cell invented a clock mechanism which allowed to anticipate the changes and to smoothen the transients.

Hypotheses 2, 3 and 4 are not restricted to the circadian rhythms of eukaryotes, and Winfree proposed to look for circadian rhythms also in colony forming eubacteria (*Actinomycetes*), Streptomycetes, tuberculosis germs, mycobacteria and nitrogen-fixing photosynthetic bacteria.

18.4 Model organisms for the evolution of circadian rhythms

Daily and and circadian rhythms are also found in fungi. As an example we got to know already *Neurospora crassa* (chapter 16). Fungi show, however, also oscillations without correlate to environmental cycles. Often the periods of these rhythms depend strongly on temperature and are synchronizable with daily light-dark-cycles. Even in *Neurospora crassa* mutants with circadian rhythms exist, the temperature compensation of which is incomplete or missing (for instance in the mutant frq9: its period is at 30°C 22 hours and at 20°C 93 hours, [922]). In this connection the following observation is of interest:

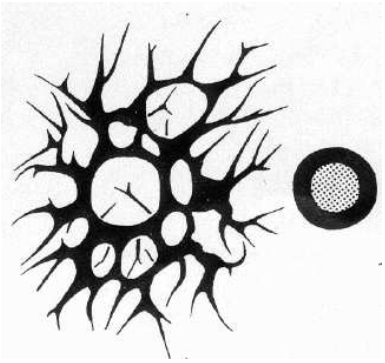

Figure 18.2: Thalassomyxa australis *changes its shape rhythmically between a resting phase (right) and an active phase with pseudopodia (left). After [527]*

[527] discovered at the western coast of Australia a marine rhizopode, which he termed *Thalassomyxa australis*. It belongs to the naked amoebae. It changes its shape rhythmically between a resting phase, in which it lies like a hut on the substrate, and a phase, in which it crawls with pseudopodia over the substrate. During this stage it takes up unicellular marine algae and digests them (section 18.2). The movie "Der Formwechsel von *Thalassomyxa australis* (Promycetozoidae)" shows its biology and this change in shape ([528]). At a temperature of 22°C period length amounts to 25 hours. At lower temperatures the period is significantly lengthened. At 10°C for instance it is 90 hours, at28°C only 18 hours ([1383]). The rhyth-

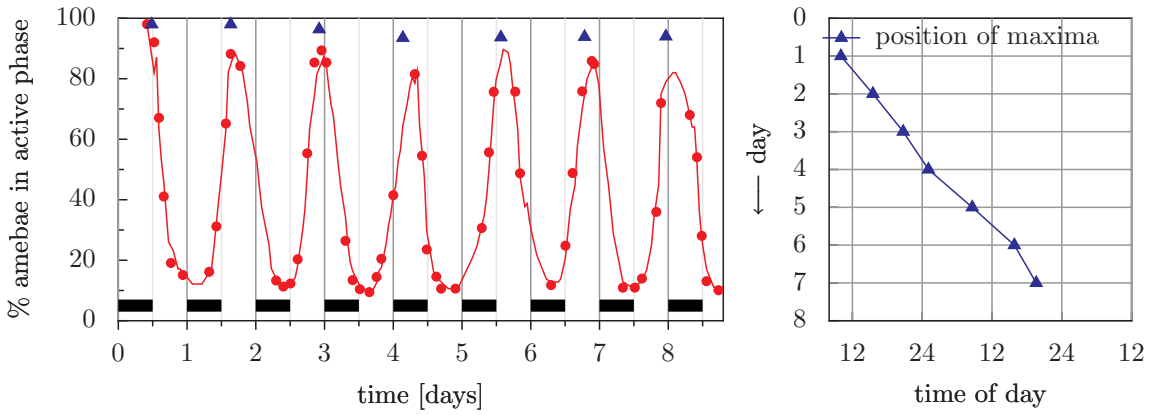

Figure 18.4: *Red circles and red curve in left part of figure show percentage of* Thalassomyxa *amoeba in active phase, with maxima as open triangles (x-axis: days). The rhythmic change in shape of* Thalassomyxa australis *can not be synchronized by a 12:12 hour light-dark-cycle (see position of maxima (triangles) to bright/gray areas). This is shown also in the right part where the maxima are seen to occur each day by 5 hours later. If synchronized, the maxima should show up in a vertical line. After [446], [1393]*

mic change in shape of this organism is not yet temperature-compensated (section 18.3). Temperature compensation is regarded to be a characteristic property of circadian rhythms. Synchronization by a light-dark-cycle and by a temperature-cycle do not function in the usual kind (figure 18.4, [446], [1393]).

Shaking of the culture (as it occurs in the ocean by the tides) synchronizes partly; it is therefore a weak time cue ([446]). Combined time cues (temperature change, light-dark-cycle, periodic shaking which simulates low tide/high tide conditions) synchronizes (18.5). It is unknown which time cues are effective in nature. We might be dealing with here a precursor of circadian clocks, a kind of Ur-Uhr (ancient clock), which does not yet possess all characteristic properties of 'modern' circadian clocks. Whether and in which way they are derived from ultradian rhythms, which possess or do not yet possess a

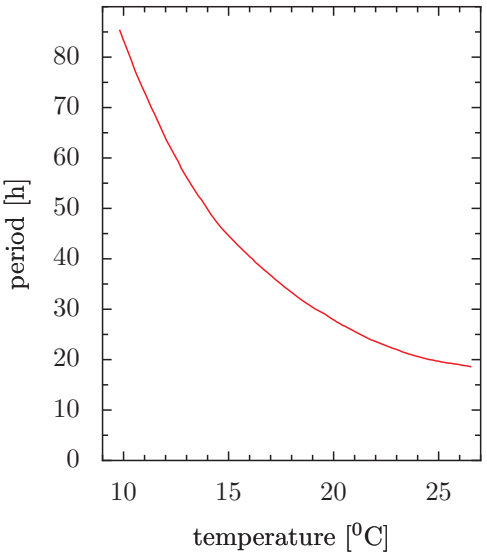

Figure 18.3: *The cycle between an active phase and a resting phase of* Thalassomyxa australis *depends on the temperature of the sea water. On the y-axis the period length, on the x-axis the temperature of the sea water are plotted, at which the amoebae were kept. After [1383]*

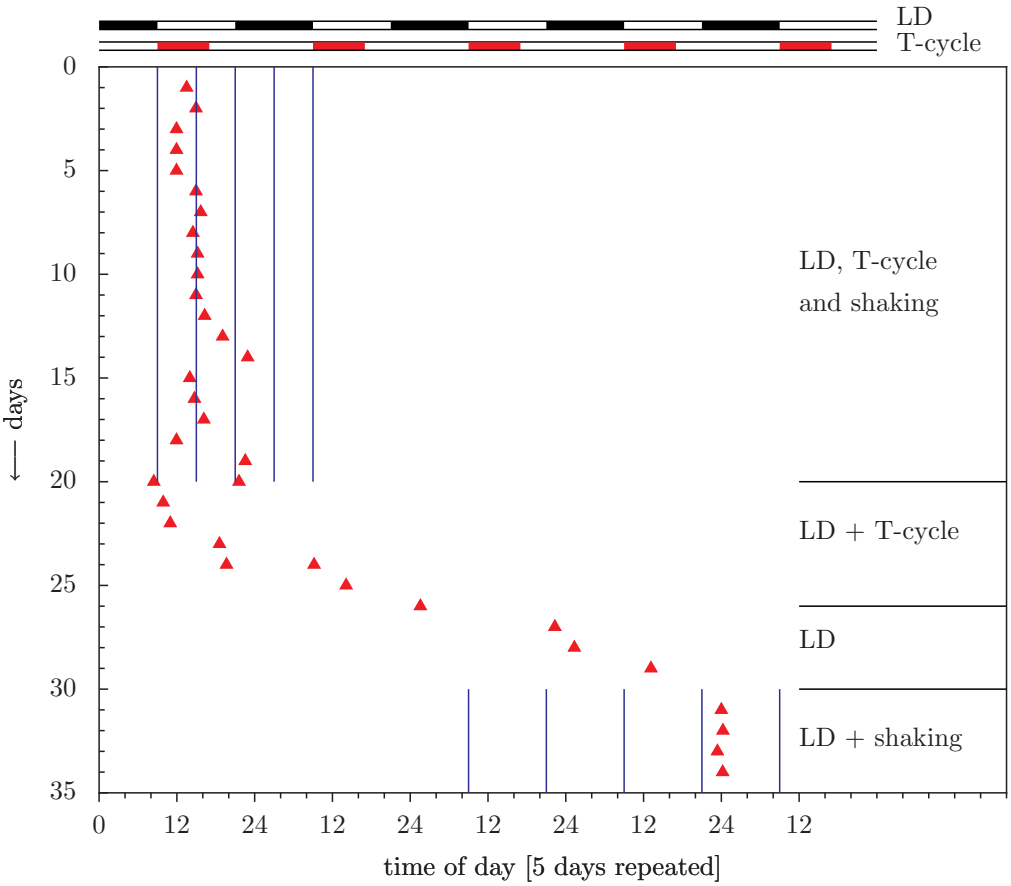

Figure 18.5: *Combined time cues (light-dark-cycle, temperature-change and shaking in a 6-hours-measure, upper part of figure) synchronize the rhythmic change in shape of* Thalassomyxa australis*, whereas neither the combination of a light-dark cycle and a temperature cycle (LD and T cycle) nor a light dark cycle alone (LD) synchronize the rhythm. However, shaking combined with a light-dark cycle synchronize. After [446]*

temperature compensation is speculative ([1382]).

18.5 Evolution of temperature compensation of circadian rhythms

In typical circadian rhythms the length of the period is almost independent of the environmental temperature. For it a mechanism is responsible, which has not been completely cleared up. A number of models have been put forward which were already presented (sections 16.10, 16.4). Of interest are more recent findings in *Neurospora crassa* ([905]). According to them the frq-gene is transcribed according to the temperature in different ways. At high temperature more sFRQ-proteins are made, at lower temperature more lFRQ-proteins. The final effect is that at different temperatures the period length of the circadian rhythm is the same.

18.6 Organization of circadian rhythms and their evolution

The mechanisms underlying circadian rhythms are much more complicated as assumed before. Feedback coupling of gene products upon the own transcription seems to be the basis of all circadian rhythms studied so far (*Drosophila*, mouse, *Neurospora*, *Arabidopsis*, *Cyanobacteria*). However, they might have evolved independently in different kingdoms ([1599]). The tooth wheels of the clock work do diverge heavily.

For instance, the proteins of *Cyanobacteria*, which serve as parts of the clockwork in the transcription of the kai-genes by negative feedback, are completely different from the clockwork of *Drosophila* and mammals (the latter, however, are homologous ([677])). Again other proteins are used in the circadian system of *Arabidopsis* ([523], [1311]). In addition not only *one* circadian clock controls the various events in a circadian way. In the same organism (*Drosophila*, [1166]) and even in the same cell (*Lingulodinium*, [1254]) different circadian oscillators seem to work. And they might perhaps use different mechanisms. The various circadian oscillators might be coupled with each other directly or they are synchronized to each other in a certain phase relationship via environmental signals.

[348] studied the origin and evolution of circadian clock genes in prokaryotes. The evolution of kai genes and their homologs occurred at different times in the history of the earth. They are found also in *Archaea* and *Proteobacteria*. KaiC is the oldest, kaiA the youngest gene. Major evolutionary factors were natural selection, multiple lateral transfer of genes and duplication and losses of genes. KaiC belongs to the RecA family, ATP- dependent combinases. The phylogenetic analysis of kai genes showed, that kaiA and kaiB evolved in *Cyanobacteria* only. The timeline of major events in the evolution of the clock genes is shown in figure 18.6 and is based on maximum likelyhood estimates.

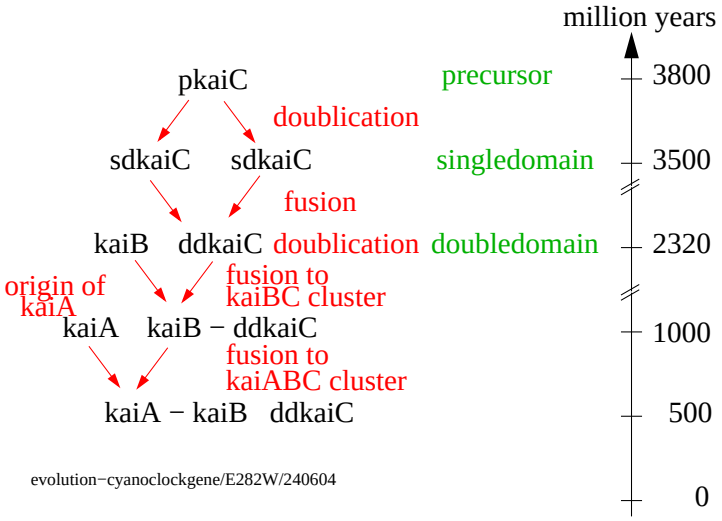

Figure 18.6: *Timeline of major events in the evolution of the clock genes of* Cyanobacteria. *Precursor pkaiC 3.8 billion years ago. Further domains originated by fusions and doublications. After [348]*

18.7 Evolution of pigment systems and photoreceptors for the synchronization of circadian rhythms

The time cues for synchronizing circadian oscillators can be quite different. Even in unicellulars (*Lingulodinium*) different pigment systems (blue and red-sensitive) affect the circadian system ([1257]). In arthropods and lower vertebrates the usual photoreceptors, but also other and/or extra-retinal photoreceptors can entrain the circadian system.

Finally there are also feedbacks from the circadian system upon the photoreceptors. For instance, the circadian system changes the sensitivity of the eyes in scorpions and beetles by several orders of magnitudes using different mechanisms ([430]). The same light of a certain intensity affects thus at different (subjective) day- and night phases the circadian system in a completely different way.

18.8 Evolution of photoperiodism

Arguments and experimental results have been presented already which showed that the photoperiodic time measurement is usually brought about by a circadian clock (see page 276). Such photoperiodic reactions might be of high selective value for the organism. They allow to adapt its development quite precisely to the most favorable times of the year. As a switch for these photoperiodic events the circadian clock can, for instance, take care that for instance an insect develops under long day conditions and enters diapause under short days.

For marine organisms photoperiodic reactions are usually less important. Here temperature changes, changes in the

biotope associated with the moon cycle and tidal cues play a larger role. Photoperiodic reactions can, however synchronize annual rhythms of marine organisms ([48], [16]).

Photoperiodic reactions became important after the land was conquered by plants and animals. Especially in the latitudes further away from the equator the time of the year has to be determined precisely in order to take measures early enough before the conditions deteriorate. Photoperiodic reactions are therefore in all organisms of the temperate and higher latitudes found independent of the environmental temperature. But even at the equator there are organisms which react photoperiodically. They have to measure daylength very precisely since they change during the course of the year only slightly. In some rice varieties differences of just one minute can induce flowering photoperiodically ([1510]). Calendar birds arrive in their breeding grounds at certain days of the years which might vary by a few days only ([72]).

18.9 Convergent development of circadian rhythms?

At the present stage of our knowledge one can speculate only how circadian rhythms might have evolved. As long as the mechanisms of circadian clocks is unknown we are not able to clarify the question, whether these clocks developed in a convergent way in the different kingdoms of organisms. The first results are now available which cast some light on the clock works. In the different systems studied so far the mechanisms use the same principle: Transcriptional products inhibit their own synthesis (mRNA formation). Time delays in these feedback cycles bring about the circadian period. But the clockwork seems to be different in the various groups of organisms (*Cyanobacteria*, *Arabidopsis*, animals) ([677], [1311], [1599]).

18.10 How, when and why did circadian rhythms evolve?

Our galaxy was formed 12 billion years ago, the sun 5 billion years ago, the earth 4.6 billion years ago.

4.0 to 3.5 billion years ago the atmosphere of the earth consisted of free hydrogen and the temperatures were 130^0C. Then nitrogen, NH_3, CO_2, CO, CH_4, and H_2S occurred. About 3.5 billion years ago the CO_2 content increased and out of a kind of 'primary soup' the first organisms evolved. Metabolism and reproduction developed. From these protobiots the eobiotes evolved, from these the prokaryotes, and, finally, about 1.5 billion years ago, eukaryots (see figure 18.7).

Eukaryots evolved, according to the endosymbiont hypothesis, by a symbiosis between archaebacteria (thermoplasma) and prokaryotes. The latter evolved to plastids and mitochondria. This was, however, a late step in the evolution of the organisms and occurred about 1.2 billion years ago. Prokaryotes evolved about 3.5 billion years ago. 1.8 to 1.4 billion years ago the oxygen content of the atmosphere increased due to the photosynthesis of organisms. The earth was thus surrounded by an ozone shield against ultraviolet light.

How circadian rhythms evolved, is not

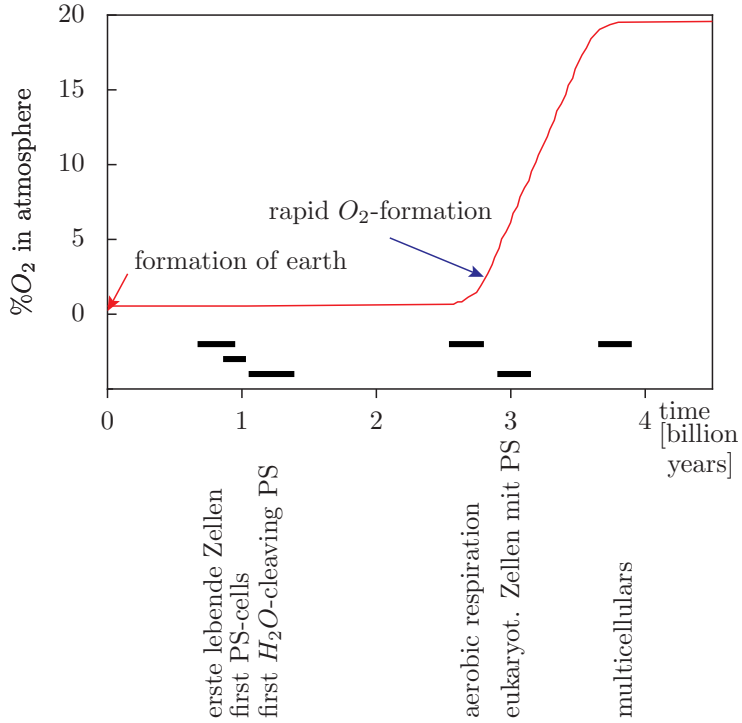

Figure 18.7: *Timetable of the evolution of the organisms from protobiots via eobiots to prokaryotes and eukaryotes. Formation of the earth about 4.5 billion years ago. First living cells about 0.75 billion years after earth formation. First water splitting photosynthesis releases* O_2. *After* Fe^{2+} *in oceans is used up, start of rapid* O_2 *accumulation. Aerobic respiration becomes widespread. 1.5 billion years ago origin of eukaryotic photosynthetic cells. First multicellular plants and animals about 700 million years ago. After [6]*

known. Organisms possess many feedback loops in their metabolism and therefore tend to oscillate, as is true for all complicated structures. It is likely, that these rhythmic events were in the begin not correlated with periodic changes in the environment. It is known, that rhythmic processes in the network of biochemical paths and in the energy metabolism are more efficient as compared to those which run with constant speed ([1060]). This knowledge is in the meantime also used by the chemical industry: At certain reactions the concentration of reaction partners is changed cyclically, thus increasing the yield.

Certainly organisms possessed a wealth of rhythmically occurring processes. If their period lengths, due to time delays, were brought into the range of 24 hours and if they could be synchronized by time cues to the change of day and night, they had a decisive advantage: They could adapt to the daily changes in light and temperature already before the onset of light. Thus, the switch-over of the metabolism to photosynthesis could occur already before the light period began. Furthermore, organisms with a circadian clock could calculate the daily course of the sun and orient themselves in this way according to the sun. With the help of a circadian clock an organisms could also measure the length of a day (or of the night) and in this way determine the season (see chapter 13).

More important was probably the protection by damaging influences of light: with the ever increasing concentration of oxygen in the atmosphere gene induction and DNA replication could easily be damaged due to photo-oxidation. This could be prevented by the organisms by quenching the excited state. Or, alternatively, the light sensitive processes occurred during the dark period. The presence of a daily rhythm would offer a decisive advantage ([1161], [1160]). On the other hand, according to [776], the symbiosis of prokaryot and host needed the coordination of the metabolisms of both partners. Furthermore, the division of the partners had to be synchronized. This was, according to the author, brought about by an oscillator. Ca^{2+} served as an intracellular signal transducer in this process (see figure 18.8). The synchronization of this oscillator by the light dark change and the temperature change occurred late in the course of evolution.

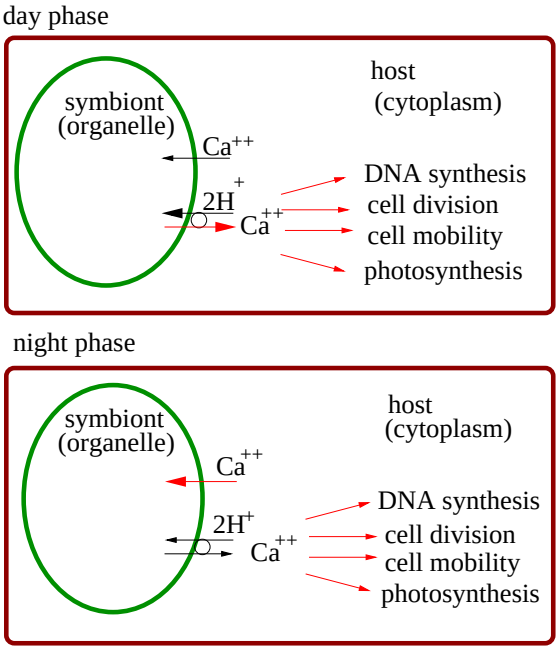

Figure 18.8: *Kippert-model of the evolution of circadian rhythms. Internal timekeeping by temporal coordination of host cell and intracellular symbionts (or, later in evolution, of cytoplasm and organelle such as the mitochondrium) in a rhythmic fashion. Still later during evolution synchrony with periodically fluctuating environmental factors such as the light-dark-cycle (day-phase, night-phase) was established. Ca^{2+} influx and efflux into and from symbiont respectively organelle plays decisive role in temporal coordination and in important cellular events such as DNA synthesis, cell division and mobility, photosynthesis. After [776]*

19 Quo vadis? The future of chronobiology

In the area of chronobiology a number of new and exciting results have been reported recently. For instance studies on circadian rhythms in *Cyanobacteria*. They cast new light also on the evolution of circadian rhythms. Circadian rhythms have apparently evolved much earlier as assumed until now. By using circadian mutants in these prokaryotes and molecular biological methods, considerable progress in the coming years has to be expected, as was the case already in the standard-objects *Drosophila*, *Neurospora, Chlamydomonas* and mice. The mechanisms of these circadian rhythms will be known better and better, but also the the way peripheric events are controlled by these rhythms. Models can help to understand the circadian system. It is likely that a concerted and concentrated effort to clarify circadian rhythms will soon lead to first successes. Minimal systems with simple structures and functions such as unicellulars and prokaryotes are especially suited. The most promising approach is to use clock-mutants and new techniques of molecular genetics, to find out the mechanism of circadian clocks in these minimal systems. If parts of the clock are known in this particular case on the transcriptional and translational level, it should not be too difficult, to see whether in other cases the circadian clocks use the same principle. The systematics and perhaps even the evolution of circadian rhythms could be clarified.

Especially important are answers to questions concerning the localization of the centers in which circadian rhythms are produced in animals. How many of these centers exist? What are the physiological and molecular biological mechanisms, according to which they function? How is the circadian information coded and how do they affect the physiological systems which they control? How are the circadian systems synchronized? How are the circadian pacemaker coupled with each other?

Furthermore, the field of chronobiology will in the years to come help to answer questions which arise from shift work and jet-lag and from medical problems such as sleep disorders.

For the progress in this area a close cooperation and intensive communication between the research groups is important. You might like to join the Clock-Club which has been formed at the end of the 'Schwerpunkt-Programm´ of the German Research Association by the participants (see clockclub moderator <clockclub-owner@yahoogroups.com>).

20 Special topics

In this chapter different special stories are collected which might also be helpful as topics of seminars. Proposals for other topics are welcomed.

20.1 Circumnutation in plants

If plants grow upward, normally elongation does not occur uniformly distributed over the cross section. Instead the growth zone migrates in a circular way. This leads, seen from above, to circular, elliptic or pendulum like movements of the corresponding tip of the organ. Seen from the side the tip of the organ describes a helix. In tendrils of climbers these circumnutations are especially pronounced. The plants try in this way to find a hold. Circumnutations are wide spread among climbing and non-climbing dicots, monocots, gymnosperms and even among fungi and bacteria. Roots can show these nutational movements too ('root waving' [1384]). Overviews are given by [42], [712], [168].

Circumnutations are always correlated with growth. At a growth rate below 0.5 mm/h no circumnutation occurs in *Periploca graeca* ([993]). The period length of circumnutations varies with temperature (Q_{10} of 2). Depending on the object it is normally between 15 minutes and 5 hours. Some species possess circumnutations with different frequencies (*Sicyos*, *Passiflora* ([520]), *Phaseolus* ([590]), *Arabidopsis* ([1341]). The excursions are just fractions of millimeters in *Arabidopsis*, but up to 1.5 m in *Hoya carnosa*. Depending on species and oscillation there are preferred directions to the right or left ('chirality'). Oscillations with different periods can overlap each other.

In the following we will deal with more closely the circumnutation of *Arabidopsis thaliana*. Which are the underlying mechanisms of these circumnutations?

To find out we have to understand first the elongation of the cells and the growth of the cell walls. *Arabidopsis thaliana* seedlings elongate quite strongly if kept in the dark or in weak light. Already in the seedling of the seeds all cells (about 20) are present, which after germination will form the hypocotyl. In the first three days after germination only the basal cells extend. Afterward elongation of the hypocotyl proceeds mainly in the central and upper part of the hypocotyl without cell division ([487], figure 20.1). However, the cells become polyploid during extension: The chromosome set multiplies without division of the nucleus and the cell.

How do the cells elongate? The wall of the outer hypocotyl cells is quite rigid and stabilizes the seedling. If cells are going to extend, their walls have to be loosened first. Afterward the vacuoles of the cells can increase in size due to their turgor pressure. New material is deposited into the walls, the cells extend and the hypocotyl elongates. The diameter of the cells stays constant. This is established by microfibrils of cellulose: They are arranged according to the principle used in

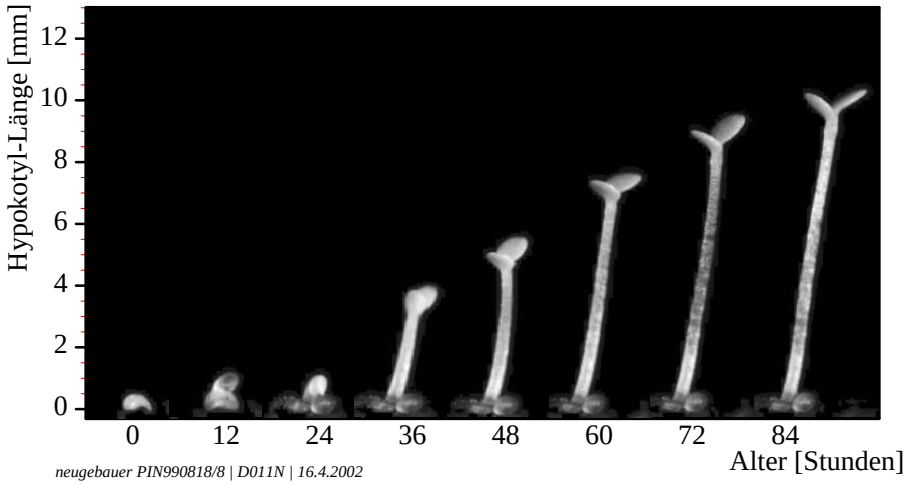

Figure 20.1: *Elongation of the hypocotyl of* Arabidopsis thaliana *from seed germination to 3.5 day old seedling in darkness. Imaging method after [1070]. Html-version shows elongation as time lapse animation (one image per 4 minutes)*

girdle tires in a circular way. The cellulose microfibrils can be pushed away from each other during volume increase of the cell (figure 1.18, events in the cell wall see figure 20.3). New cellulose fibrils can be inserted as rings, but the diameter of the cells stays constant.[1] In this way the increase in volume is used only for extension of the cells. The hypocotyl can grow as fast as possible upward in order to reach the light which is so essential for the plant.

In order that elongation can occur the contact points in the network of the cell wall have to be loosened. The wall consists according to a model of [260] (figure 20.2) of three nets with different structure, which are interwoven with each other. They provide the wall with strength, flexibility and the ability to increase in size. A 'cell wall loosening factor' is responsible for the plastic properties of the wall. According to the acid growth hypothesis of [547] this factor was supposed to be protons. Plasma membrane-bound ATPases are activated by auxin and its concentration increased. They pump protons in the cell walls, which become in this way plastic. In the meantime it was, however, shown, that expansin, a protein (figure 20.3), loosens the walls ([258]). How this might occur is shown in figure 20.4. Depending on whether receptors for expansin in the cells are active or not, the cell reacts to this protein.

The exact role auxin plays is not yet fully understood. Elongation is at least promoted by auxin.[2] Auxin is synthe-

[1] Or the cell wall material parts from each other and the microfibrils turn from transverse to longitudinal ([1243]).

[2] Auxin has a rapid effect which starts already after 8 minutes, reaches its maximum after an hour and is over after 2-3 hours. It lowers the pH, activates the phospholipase, sets in motion a cascade of phosphorylations and activates protein kinases.

Furthermore auxin has also a slow effect. It induces processes which are needed for elongation. Osmoregulation and gene activations of

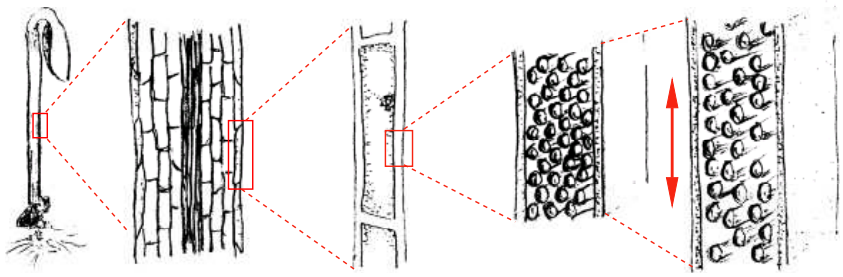

Figure 20.2: *Structure of hypocotyl of* Arabidopsis *seedling. From left to right: From a seedling a part of the hypocotyl is shown enlarged. Of this, a single epidermis cell is depicted. A short piece of its wall is schematically drawn in the original state (second last image) and during wall extension (last image at the right). The cellulose fibrils are by cell pressure and other events (see figure 20.4) pushed away from each other, before new cellulose fibrils are formed and inserted (from the terminal complexes in the plasma membrane). According to [255] and [256]*

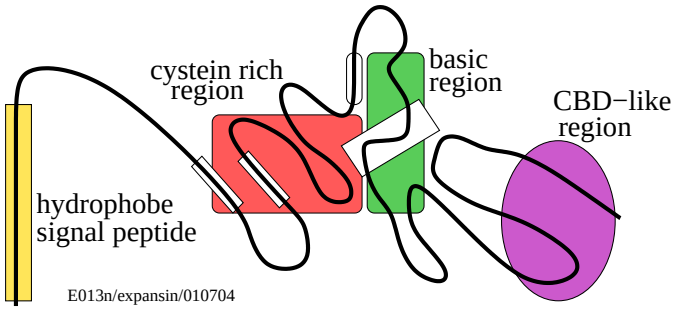

Figure 20.3: *Structure and amino acid sequence of expansin S1 of pumpkins. The following properties are highlighted: A hydophobic signal peptide at the amino-end (left), a central region (red) with eight conserved cysteins. Some of these form probably intra-molecular bridges. One region with several conserved basic residues (green). One region at the carboxylic end with four conserved tryptophanes (purple). This region is somewhat similar to the cellulose-binding-domain of bacteria (CBD). Aspartic acid residues located here are perhaps determining the acid-optimum of the expansin-activity. After [259]*

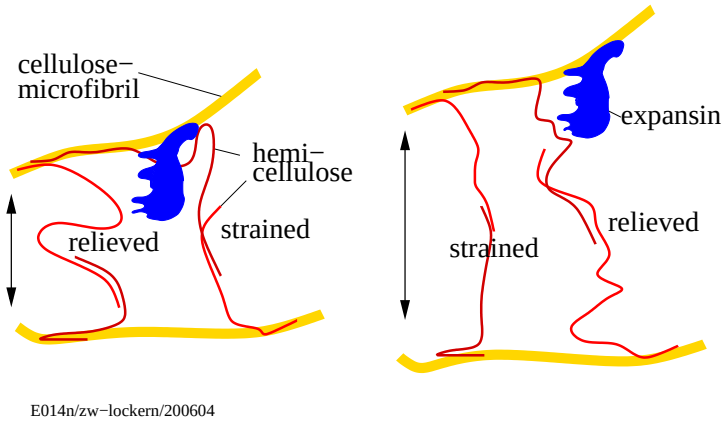

Figure 20.4: *The cell wall is loosened by expansin (blue) which loosens the connection between cellulose-microfibrils (yellow) and the hemicellulose (light blue, red). The microfibrils can now be pushed away from each other by the wall stress, the polymer 'creeps'. New cellulose-microfibrils are formed at terminal complexes and new hemicellulose is inserted and connected (not shown). Other wall components are also extended (not shown). After [257]*

sized in the apex and transported in pulses to the sub-apical region. The transport occurs via the phloem or polar via the parenchyma and xylem. The transport speed is 5 to 20 mm/hour. In the plasmalemma of the cells a carrier for the auxin transport is needed.

How do circumnutations arise? We have seen that the hypocotyl-cells do not uniformly elongate. At the onset of germination the cells at the base elongate (they are sensitive to expansin at that time). Later the cells in the central and upper region of the hypocotyl increase in length. The cells at the base are now insensitive toward expansin. But elongation does not even occur uniformly in the same growth zone. Instead one side of the hypocotyl reacts with extending, and thereafter the neighboring cells extend. In this way a wave of elongation runs around the corresponding hypocotyl section. As a result the tip of the hypocotyl turns. Thus on the level of organs periodic changes in the osmotic potential of the epidermis cells lead to plastic and elastic extension. In the part where the hypocotyl is bending, K^+ is distributed asymmetrically and ring-like (figure 20.5 *Phaseolus* [39]). Growth is thus occurring in fits and starts both in time and in space. The hypocotyl of *Arabidopsis thaliana* shows a whole spectrum of circumnutations: It consists of 'short period nutations' (SPN) with period lengths of about 30 minutes, of 'long period nutations' (LPN) with period lengths in the hour range, and occasional 'ultrashort period nutations' (uSPN) of about 15 minutes. SPN and LPN differ by a number of properties which are put together in table 20.1. Additionally the two kinds of circumnutations differ also in their reaction toward light and ethylene. It is therefore assumed that different mechanisms are responsible and/or different parts of the hypocotyl are taking part. SPN´s are mainly found after germination when the

protein- and carbohydrate synthesis pathways belong to it.

Table 20.1: *Different properties of the various circumnutations*

property	SPN	LPN
period length	20-60 min	1-8 h
deviation	40 min	> 7 h
distribution	one peak	several peaks
preferred direction	clockwise	counterclockwise
correlation with growth	strong growth	slow growth

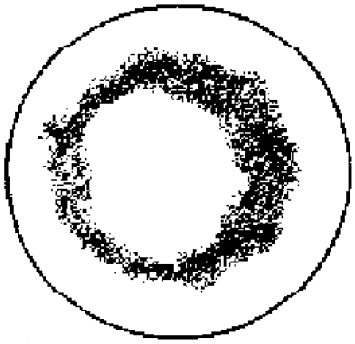

Figure 20.5: *In the part where the hypocotyl of* Phaseolus *is bending,* K^+ *is distributed asymmetrically and ring-like. After [39]*

hypocotyl begins to extend ([1341]). In the first three days only the basal cells elongate ([487]). Perhaps the SPN's are the result of the circulating elongation growth of the basal hypocotyl cells, whereas the LPN's are the result of the later occurring extension of the cells in the central part of the hypocotyl. This should be testable by marking the different parts of the hypocotyl.

The growth of the hypocotyl of *Arabidopsis thaliana* is furthermore under circadian control, as shown under constant conditions. There are times with small growth rates and times with high growth rates. Since ultradian circumnutations occur only during growth, they must be modulated by the circadian rhythm: The amplitudes of circumnutations increase correspondingly, reach a maximum and disappear again. After one circadian cycle they reappear again. In *Arabidopsis thaliana* there are other events which are controlled in a circadian way ([1009]).

Growth and circumnutations of *Arabidopsis thaliana* (and other plants) can be recorded and analyzed by using imaging procedures. For this purpose the seedlings are observed with two videocameras from the side or from above and pictures are taken at regular intervals and stored. The images can be evaluated with special procedures and programs ([1070]).

20.2 Biological significance of the REM-sleep

REM-sleep is only found in mammals and birds. It is especially well developed in the higher mammals. Reptiles do not have REM-sleep. In contrast to man and apes small mammals and birds show only two sleep stages. One of it is similar to slow wave sleep 4, the other to REM. The ant eater is the only mammal without REM-sleep.

The paradoxical sleep or REM-sleep seems to be a primitive form of sleep. The deep sleep is phyllogenetically a recent invention. REM-sleep is to a large extent controlled by circadian factors (in addition

to a homeostatic control, [1585]) and exerts a crude regulation only. Sleep deprivation is for instance without effect on REM-sleep. Only after massive deprivation it will be prolonged. The areas in the basal brain, which are responsible for the REM-sleep, are phylogenetically very old. REM sleep occurs already at an early stage of development (see page 418).

We dream each night several times in regular cycles. Dreams are, however quickly forgotten: A REM-sleep-dream can not be recalled after 8 minutes. This is the reason we remember mainly dreams from the morning. The physiological intensity of the REM-phases increases with the passing night and therefore those dreams which are the most emotional one with much visual content are in the morning.

Eye movements are indicators for the REM-sleep. Neuronal spike-mechanisms (PGO-spikes) occur parallel to the dreams, especially to those with event-loaded contents. They cause probably REM and dreams. Similarly the penis-erections which occur at the begin of REM-sleeps are most likely caused by PGO's. Dreams occur, however, not only during REM-sleep, as assumed before, but also during the deep sleep.

How is the REM-sleep brought about and where is it localized? According to [631] there are certain areas in the brain, parasympatic and symphatic ones, the stimulation of which (4-6Hz, 2.5-3.5V) induces sleep or wakefulness. Thalamus and hypothalamus, but also other centers, even peripheric nerves are involved. Hypothalamus-lesions in the cat induce sleep. A second sleep center in the pons seems to be responsible for the REM-sleep. There is finally a mesencephalic wake center and antagonistic centers.

[724] was able to influence the REM-sleep of cats by pons lesions in such a way, that the inhibition of the large muscles (atonia) was abolished and the animals were able to execute their 'behavior in dream sleep'. Juvet assumed that the REM-sleep serves for programming of species specific behavioral patterns and for exercising. Therefore they function already the first time they are occurring. The neuronal circuitry for this instinctive behavior is, according to this author, organized during the REM-stage. In cold blooded animals these behavioral patterns are already pre-programmed and therefore they do not need REM-sleep. Against it speaks, that these patterns can develop also in other ways, for instance during play. Furthermore, the behavior depends on the position and size of the lesion, which was done to abolish atonia. Aggressive behavior is induced, if the lesion is extended more rostroventraly into the middle brain. The animals are after such a lesion aggressive also during the waking state. That is, it is a non specific change of the behavior. Important is the finding, that during REM-sleep atonia occurs which is abolished by lesions.

What is the biological function of the REM-sleep? If test persons are woken up each time the REM-sleep starts, they are deprived of most of this type of sleep. This interference has, however, no influence on the SWS. The REM-sleep is in the nights after REM sleep deprivation earlier, longer and more frequent. There are apparently no serious psychological disturbances even after 16 days of REM-sleep deprivation. The REM-sleep rebound after deprivation shows, however, that this sleep stage is physiologically necessary. It furthermore shows, that dreaming is important, because dreams occur

during this stage (but not exclusively, see section 2.3). Chronic treatment of certain diseases with monoamine oxydase-inhibitors prevent REM-sleep and dreaming for years. There are apparently no detrimental psychological and physiological consequences (discussed by [726]).

What happens during the REM-sleep? Different hypotheses exist. According to one of it certain processes are programmed in the brain in this stage as for instance instinctive behavioral routines or the practicing of events. According to another hypothesis recovery processes take place in this stage. A further hypothesis claims that REM-sleep has a guarding function: The environment is periodically checked. This is known from birds. In mammals the REM sleep would then be a relict from the reptiles without function. Unfortunately these hypotheses are difficult to test.

[616] claim that the REM sleep consolidates memory performance. Experiences are taken up into the memory. The main difference between the wakefulness stage and REM is the different weighting of sensory afferents in cognitive images. Otherwise the two stages are equivalent brain states which are produced by thalamocortical feedback.

According to [1259] REM-sleep plays for the developing brain a similar role as physical exertion plays for the development of the muscles. During REM-sleep neuronal circuits are strongly activated. During this stage more oxygen is consumed in the brain as during physical or mental exercises in the wakefulness stage. It was therefore presumed, that the REM-sleep is important for the development of the brain ([508]).

[268] speculate on the function of the REM sleep. According to them dreaming during REM sleep is a way of an active de-learning. Useless or damaging informations are discarded in a kind of self-cleaning process. The contents of the dreams are more or less products of chance. It is therefore better to forget dreams. Otherwise one would take them for true, as is the case in madness.

[152] propose that during REM sleep transfer of visual informations between the visual cortices and the limbic system are activated, but transfer of visual informations to the pre-frontal association cortices are not. As a consequence, the extrastriate cortices and the paralimbic areas to which they project may operate as a closed system, dissociated from frontal regions. The brain engineers in this stage a selective activity of an inter-optic network. It is dissociated from primary and heteromodal associated areas at either end of the visual hierarchy that mediates interaction with the external world.

The cortex of birds and mammals (with the exception of egg laying, such as ant eater) consists of a network of cells with a large variety of states of mutual stimulations. During the storage of several memory items parasitic conditions can easily occur:

- If nets which have nothing or almost nothing to do with each other are connected, bizarre associations or fantasies arise.
- If many individual nets are stimulated, one and the same large net is stimulated. Possession and fixed ideas are the result.
- If signals lead to stimulations which should not stimulate, hallucinations result.

Epilepsy and migraine might also be the result of such parasitic conditions.

20.3 Ontogeny of the circadian system of man

The temperature rhythm is at birth only weakly expressed. It develops in the first year of life. When it attains the amplitude of the grownup is unknown. In aged people deficits of temperature-regulation occur. The period length shortens from an average of 25.1 to an average of 24.5 hours. The phase relationship changes and the amplitude decreases.

The sleep pattern changes drastically during the development. In newborns the sleep is polyphasic, in children biphasic and in the adult usually monophasic. It is synchronized to the day-night-cycle.

Newborns sleep 16 hours per day. They exhibit a rest-activity-cycle of 50-60 minutes. It soon couples with a digestive cycle with 3-4 hour periods ([1030]). A 24 hour rhythm develops with about 18 weeks[3]. From 6 months onward they sleep for 10 hours, and day sleep is less frequent. With two years children sleep 13-14 hours per day. The sleep requirement decreases during childhood and adulthood, stays constant in the middle age, and decreases again in old age. The strongest changes occur in REM-sleep and in stage 4 of the slow wave sleep. The REM-sleep occurs already in the fetus in the uterus. Prematurely born babies are by 80% (10 weeks earlier born), 60-65% (2-4 weeks earlier born) of the whole sleep in REM stage as compared to 50% (normal time of birth). With 2 years this value decreases to 30-35% and with 10 years to 25%. This stays so until the 70th to 80th year of life. The stage 4 of the slow wave sleep decreases exponentially during the years of developing and during the middle ages. With 60 years this stage disappears often completely, which is often connected with frequent spontaneous awakening and biphasic sleep.

Further informations in [295], [990], [988], [989], [502], [1260]

[3] There might be cultural or geographic differences. In Japanese and Indian babies the daily rhythm of sleep is already recognizable immediately after birth ([1481], personal communication and data from Marimuthu) .

20.4 Chronobiological phase type

This questionnaire is designed to determine your chronobiological phase type. It is concerned with your activities and how much you feel awake in the morning and in the evening. In answering questions 1 to 4: Assume that you can work eight hours per day at times you are free to choose. Answer all questions. Cross only one answer. Be honest.

1. How difficult is it for you if you have to go to bed each day at 1:00 o'clock
   - (4) Very difficult. I would be terribly tired for a long time
   - (3) Quite difficult. I would be tired for some time
   - (2) Not difficult. I would feel slightly tired
   - (1) Not difficult, no problem
2. How difficult is it for you if you have to rise up each day at 6:00 o'clock?
   - (1) Very difficult. I would be terribly tired for a long time
   - (2) Quite difficult. I would be tired for some time
   - (3) Not difficult. I would feel slightly tired
   - (4) Not difficult, no problem
3. You have decided to participate in a fitness-training. Your friend proposes to train twice per week. For him/her the best time would be from 7 to 8 in the evening. How would this be for you?
   - (4) It would be optimal
   - (3) would be all right
   - (2)I would have difficulties, I would prefer a later time
   - (1) It would be to hard for me
4. You have decided to participate in a fitness-training. Your friend proposes to train twice per week. For him/her the best time would be from 23 to24 in the morning. How would this be for you?
   - (4) It would be optimal
   - (3) would be all right
   - (2)I would have difficulties, I would prefer a later time
   - (1) It would be to hard for me
5. Mark the time span in which you *normally* go to bed. The uppermost row is an example for somebody who normally goes to bed between 22:00 and 23:30.

example ——— –											
20		21		22		23		24		01	02	
(5)–>				(4)–>		(3)–>		(2)–>		(1)–>		

6. Mark the time span in which you *normally* wake up.

———											
05		06		07		08		09		10	11	
(5)–>				(4)–>		(3)–>		(2)–>		(1)–>		

7. Are you a morning- or evening active person?
    - (5) extremely morning active
    - (4) moderately morning active
    - (3) neither
    - (2) moderately evening active
    - (1) extremely evening active

The values in parenthesis should be summed up (in the examples of question 6 one would use (4) and not (5)). The chronobiological phase type can be determined using the sum of the scores in the following:

- 7-10 extreme evening type
- 11-14 evening type
- 15-21 indifference type
- 22-25 morning type
- 26-31 extreme morning type

20.5 Circadian rhythms in the blind

Here some more recent original publications are compiled.

At the begin of the studies of the circadian system of man it was assumed, that the light-dark -cycle does not play a role in synchronization (studies in a subterranean apartment in Erling-Andechs south of München). Later it turned out that the relative low light intensities in the rooms were the reason for this assumption. If intensity is increased above 2500 lux, the circadian system is synchronized as is known from other vertebrates. The light-dark cycle plays thus an important role also in the synchronization of circadian rhythms of man. However, higher intensities are needed as compared to most of the animals studied in this respect.[4] A further hint are studies of the circadian system of blind people.

Blind people who do not perceive any light, display in many cases a free run of their circadian rhythms ([1008]). This is often the cause of sleep disturbances. Miles studied 50 blind people. 38 had sleep difficulties. 20 of them showed disturbances of the sleep-wake rhythm, one of them exhibited a free run with a period length of 24.9 hours. Such irregularities are often found, if the internal rhythm, due to its free run, is not any more in synchrony with the day-night cycle. The rhythm can be determined by measuring the endogenous melatonin-secretion at different times of the day (the concentrations of metabolites in the urine are measured).[5] The melatonin rhythm is relatively free from disturbances. In this way it can be tested whether a blind person is synchronized to the day or shows free run.

Some blind people are synchronized quite normally, perhaps by remnant ganglionic cells in the retina with intact retino-hypothalamic connections to the SCN.[6]

Exogenously applied melatonin shifts the phase of circadian rhythms of rodents and of man and synchronizes in some cases, if applied daily at a certain time (before sleep onset). It can be used in some cases of sleep disturbances in the blind, in order to synchronize sleep ([22]). Its effect is however weaker as compared to endogenous melatonin. The synchronization of other circadian rhythms by melatonin is more rarely seen ([23]).

20.6 Control of body temperature in mammals

Body temperature of fish is normally just a little bit above water temperature. Exceptions are some fish with warm bodies such as tuna and certain sharks. They possess a counter-current -vessel system in the muscles, which preserves heat of muscle

[4] Under constant conditions of weak light (8 lux) a group of 6 persons showed free run of body temperature-, activity- and sleep-wake-rhythms, although they knew the times of the day ([1007]). This indicates, that the light-dark cycle is important for synchronizing circadian rhythms.

[5] The pineal secrets melatonin diurnally during the night. The duration of secretion depends on the length of the dark period. Mammals obtain photoperiodic information for synchronizing annual rhythms by using the melatonin messages. The significance of melatonin in the circadian physiology of mammals is less clear.

[6] The melatonin-rhythm was recorded in 49 blind people. 19 of them were able to recognize light, 30 not. In most of the first group (14) the rhythm was synchronized to the 24-hour-day. In the second group most people were not synchronized (23). 17 of them showed free run, 5 were synchronized in an unusual way ([915]).

activity. In this way the body temperature can be up to 21^0C above water temperature. In the blue-fin tuna fish the eyes and the brain are warmer. Controlled heat exchange increase the efficiency of fish and the tolerance toward different water temperatures. Tuna fishes for instance can swim in waters with temperatures of 5^0 to 30^0C. They are able to cover in 50 days distances up to 6700 km. For short periods they might reach a speed of about 70 km per hour.

In terrestrial animals the temperature differences can be much larger. For instance, the temperature at the Lena river in Siberia rises in the summer to 31^0C and falls in the winter down to -71^0C.

Amphibian try to find at high air temperatures places with lower temperatures, cool the body by transpiring or stay in the water.

Reptiles prefer during the day warmer and during the night cooler places. During the winter they find protected places.

Mammals and birds keep their body temperature more or less constant in spite of strong changes in the environmental temperature (homeostasis). A more intensive metabolism produces heat. In addition the fur increases thermal insulation. This insulation is in Huskies so perfect, that they are able to sleep outdoors even at -30^0C without increasing metabolism significantly. Animals can furthermore insulate themselves against the environmental temperatures by building burrows, subterranean nests and food reserves.

A constant brain temperature is especially important in homeothermic animals. The body temperature of an antelope for instance might rise to 44^0C, but the brain temperature does not increase beyond 40.5^0C. A special heat exchange mechanism is used for accomplishing this ([46]). The temperature regulation starts to work, if the hypothalamus temperature deviates by 0.5^0C from the reference value. This regulation occurs also during torpor or during hibernation, but with a changed reference value. During the REM sleep no temperature-regulation is found. During slow wave sleep the reference value of the temperature regulation is by 2^0C lower as compared to the wake state.

What are the advantages of homeothermia? The organism is much more persistent and are able to react at cooler times of the day or the year much quicker. They have to pay for it however by a twice as high an energy demand. A further question is, why birds and mammals use such a high temperature at all. Probably at the time when mammals occurred in the course of evolution, the heat loss was the main reason: At high body temperature heat can be given off and cooling can be achieved by transpiring even at high external temperatures (panting, sweating).

The physiological basis of temperature regulation and homeostasis are briefly presented in the following (for reviews see [115], [192], [261], [617]).

20.6.1 Temperature regulation of homeothermic animals

Constant temperature means, heat production and heat loss balance each other ([115], [135] and textbooks on physiology such as [737]. Heat is mainly produced during metabolism and might increase by a factor of 10 at high activity. This is the reason, why heat discharging mechanisms are so important. Heat loss is strongly dependent on external conditions such as temperature and wind. There are three different mechanisms of heat loss:

1. Heat conduction
2. Heat radiation
3. Cooling by transpiring.

Heat conduction and heat radiation are only effective at cooler environmental temperatures. Very effective is convection with automatic flow (thermic) or induced flow (wind). The body with its 300 K temperature emits independent of skin- and fur color[7] heat in the infrared (around 10 000 nm). To cool the body transpiring is the most important mechanism. It is used in sweating and panting. For 1g of water the heat loss amounts to 580 Cal (about 140 J). In the state of heat balance the heat production of the metabolism (in the resting human about 70 kcal per hour) is balanced by the sum of the three heat loss mechanisms.

Heat production occurs to 56% in the organs of the chest and the abdomen, although they participate with 6% of the body mass only. Heat production is thus mainly in the kernel of the body and not in the periphery (skin, muscles). Due to muscle work the metabolism and thus heat production is increased by a factor of 10. To discharge the 70 kcal heat of the resting human by transpiring, 120 ml water would suffice.

Dessert animals possess special mechanisms for heat conductance. They also tolerate more heat storage (Camel: 2900 kcal, corresponding to 5l of water). The temperature gradient is correspondingly smaller, and only 1/3 of the waters is needed for cooling. The fur isolates additionally against heat and saves 50% water. In this way a camel can thirst for 6-8 days. It is afterward able to drink up to 1/3 of its body weight. In the camel the body temperature is during the day by 2^0C higher as during the night. If water deprieved, the difference is 6^0C. This saves water and energy.

How is body temperature controlled? The periphery of the body may experience relatively high temperature differences, whereas the differences in the core are only small. Aim is to keep the body temperature close to the reference value ([115]). Two control systems achieve this:

1. Thermal skin receptors in the periphery: The first defense line against cold and heat. Here we are dealing with a broad band control. It influences the peripheric circulation by the vasomotoric tonus (expanding and constricting vessels).
2. Thermal receptors in the kernel of the body (hypothalamus). It regulates sweating and coldness shivering and is a second defense line against cold and heat. Here we are dealing with a small band control.

In figure 20.6 this control system is shown schematically.

For experiments the body temperature can be measured with probes, which transmit the signals via a cable or a radio transmitter (which is intraperitoneally implanted). Infrared pyrometers are also used. Rectal temperature is usually measured, but also the tympanum temperature, which does not deviate by more than 0.2^0C from the body temperature (measured in cold-exposed persons). In analyzing the recorded time series, periodograms and frequency histograms are produced.

[7] The color of the skin and fur is however important for absorbing radiation in the visible range (where dark skin absorbs more visible light, but protects from UV.

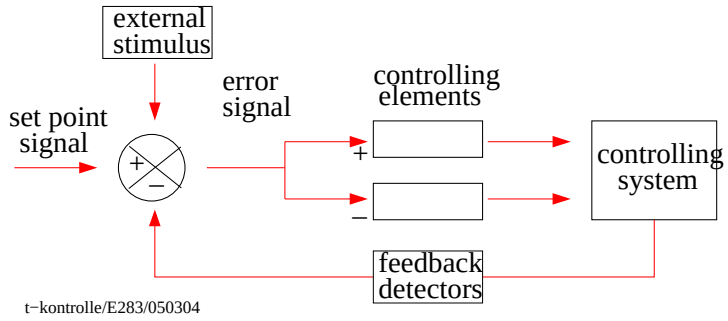

Figure 20.6: *Control system of body temperature (and of many other homeostatic processes). A set point signal (here: mean body temperature) is compared with an afferent signals from the controlled system (here: temperature receptors of skin, hypothalamus and other parts of the body). In case of differences an error signal is sent to positive (heat production) respectively negative (heat dissipation) controlling elements which affect the controlled system*

20.7 What is sleep?

Phases of activity and rest alternate not only in vertebrates, but also in lower animals such as mollusks and insects and even in Unicellulars. These phases of rest in lower animals could be the precursors of the sleep-wake rhythms in vertebrates. They are usually under the control of a circadian clock. According to [128] this circadian control of rest is, however, too rigid. Therefore a special process S is used in higher animals, which controls sleep according to the external conditions much more flexible as the rest-activity cycle does.

[1157] defined sleep as a necessary process which occurs rhythmically more or less independent on external conditions. In this stage sensory and motoric interactions of the brain with the environment are interrupted. We know, however, in the meantime that during certain stages of the sleep sensory informations from the periphery enter the cortex and motoric commands of the cortex reach the α-motor neurons of the spinal cord, although the efferences of the motor neurons are closed down. Sleep is thus not just an idling process, but an active neuronal process in which different psychophysiological events follow each other cyclically. They are controlled by different neurochemical systems. Details and the history of studying the physiology of sleep can be found in [737]. See also [127].

Sleep structure and sleep stages: Sleep of man is structured by the occurrence of different slow wave sleep stages and REM sleep. For the properties and significance of REM sleep see section 1.7.

It takes about 30 to 45 minutes after the end of the waking state to enter the deepest SWS. After this time it takes another 30 to 45 minutes to return to the flattest SWS. Afterward a REM-stage is reached, before the different SWS stages occur again. This change between SWS and REM is found about 4 to 6 times per night. With night progressing, the REM episodes become longer and the distances between them shorter. In the young adult about 25% of the sleep is REM, about 50% is SWS stage 2. Stages 3 and 4 occur mainly in the first

half of the night, the flatter SWS stages 1 and 2 and the longer REM stages in the second half of the night.

During the SWS the muscles are relaxed, but the body is still active. For instance, the position of the sleeper is changed about every 20 minutes (in some persons even in shorter periods, for instance every 5 minutes). Parasympathetic activities are predominant. Heart beat and blood pressure are lowered, the gastrointenstinal activity increased. With increasing deepness of sleep it becomes more and more difficult to wake up the sleeper.

Differences in sleep duration are compensated by the intensity of the sleep (deep sleep stages 3 and 4 of the SWS are more pronounced, 'sleep homeostasis´).

Sleep pattern: Most adults in many western countries adapted a sleep pattern in which they rest throughout the night in one stretch and do not use resting periods ('naps') during the day. There are, however, countries in which people take a siesta at the early afternoon time. It is likely, that this pattern is more adequate to the requirements of the body. It is at least recommendable to use a timely pattern ([1202]). Monkeys use 5-6 rest- and sleep periods per day.

Trigger of sleep and sleep preparations: To rock babies and to sing lullabies induces sleep in children. A relaxed atmosphere, routine work and monotony instead of intensive activities and excitement in the evening help also the adult to fall asleep more easily. Certain movies lead easily to sleep disturbances, other movies are sedative.

Frequent yawing precedes often the onset of sleep. It is important for the circulation. The body relaxes, the efficiency decreases. The latency time of conditioned reflexes increases. The eye lids close, the temperature of the feet increases, the core temperature is lowered and the muscle tonus falls. The action potentials of the muscles are reduced, heart beat and blood pressure decrease.

1-5 minutes after onset of sleep twitches occur for about 5 minutes which are caused by the cortex. The alpha-rhythm disappears and the muscle tonus decreases further.

The time to fall asleep varies with age. Students need 13 minutes as an average (8-23 minute range), Kindergarten children 36 minutes (24-64), middle aged men 15 minutes (9-25), women 13 minutes (10-17). Environmental conditions are also of influence: Less time to fall asleep is, for instance, needed at lower atmospheric pressure.

Body position during sleep: The body position is changed during the sleep every 3 to 5 minutes: In this way it is avoided that limbs become numb. The eye lids are closed, the eye balls turned upward and outward. Muscles are relaxed. Sleep positions vary a lot.

Sleep length: The length of sleep amounts to about 30% of the day in the adult as an average. There is no correlation between length of sleep and school success in children. However, children with a high IQ sleep shorter. But gifted children sleep longer. The sleep of Japanese children is shorter as compared to European children. Women sleep somewhat longer as compared to men, as an average (7.5 hours, extremes are 6 or 9 hours, observed in 25 women). Sleep

shortens with age. The season affects sleep length. Waking with the help of an alarm clock shortens sleep from 7.8 hours to 7.2 hours ([13], [667], [991], [1396], [801], [1023]). According to [1618] sleep length is to a certain degree predictable:

- by the temperature at the onset of sleep: The higher, the longer.
- by the position of the body temperature minimum: The earlier after sleep onset, the shorter.
- by the temperature increase after the minimum: the slower, the longer.

Dreaming: Everybody dreams frequently in each night in a regular cycle. However, dreams are quickly forgotten: A REM sleep dream can not be remembered after 8 minutes. This is the reason that we remember mainly dreams in the morning. The physiological intensity of the REM phases increases during the course of the night. Parallel to it the dreams are more emotional and often with visual contents. Eye movements can be used as indicators for the REM sleep. Neuronal spike-mechanisms (PGO-spikes) run parallel to the dreams, especially to those rich in events. PGO spikes are probably the common cause of REM and of dreams. Penis-erections found at the begin of the REM sleep are probably also caused by the PGO's.

Many dreams are unpleasant (64%), only 18% are pleasant or exciting. Blind people dream acoustically. The passage of time during a dream is not condensed, as often stated.

Dreams are not restricted to the REM sleep. They occur also during the SWS. They are, however, less well remembered, are less emotional and of less visual content. Instead, they are more conceptual and plausible. As a rule they are more pleasant. Exceptions are night mares which occasionally occur during the SWS 3 and 4. They are combined with dyspnea, paralysis and anxiety.

Sleep hypotheses: A number of hypotheses were proposed, which deal with the physiological basis of sleep. Some of them have been tested experimentally.

- For instance sleep inducing substances were found. [1157] has taken cerebrospinal fluid from a dog which was sleep deprived for several days. The fluid was injected in another dog which slept for 6 hours afterward.
  Later quite a number of substances were found which induced likewise sleep: Muramyl peptide, lipopolysaccharides, prostaglandine, interleucin-1, interferon alpha_2, delta-sleep-inducing peptide, vasoactive intestinal peptide and serotonin. All of them did not only induce sleep, but affected also body temperature and interacted with the immune system.
- [631] could induce sleep in cats by stimulating electrically certain parasympatic and symphatic brain areas (4-6Hz, 2.5-3.5V). The effect depended on the kind and strength of the stimulation. Thalamus and hypothalamus were involved, but also other centers and even peripheric nerves. Important is only the kind of stimulation. But certain sleep- (and wake-) centers seem to be involved: Lesions in the hypothalamus induced sleep. Another sleep center was found in the pons, which induced

REM sleep. Furthermore a mesencephalic wake center was located. The sleep- and wake centers are apparently antagonistic.
A non-specific ascending reticular activating system (ARAS) seems to exist and a diffuse thalamic projection system (DTPS). If the ARAS is active, wakefulness is induced and sleep terminated. DTPS induces the alpha rhythm and maintains a certain state of excitation. Basis of the short-term activity rest cycles (BRAC) seems to be a change between LVFA sleep phases and HVSA[8].
Finally the *carotis sinus* is significant for sleep induction.

- Humoral sleep hypothesis ([1118]). Toxins are supposed to be responsible for the induction of sleep. However, sleep deprivation does not increase sleepiness gradually, but in waves. Furthermore the efficiency is low in the morning, that is, at a time where the concentration of the toxin should be low. In any case hormone concentrations do change with the sleep-wake-cycle. See ([1616]) for examples.

- According to [725] the neurotransmitters serotonin and catecholamines as counterplayers are responsible for sleep induction and wakefulness.

- Kleitman proposed a developmental hypothesis for sleep: A primitive sleep-wake system exists sub-cortical. It is without dreaming and found in newborn babies and occasionally in children without cortex. The cerebral cortex is dispensable for sleep to occur in dogs too (cerebral cortex is dispensable for sleep to occur in dogs too ([509]). It consists of a basic rest-activity cycle (BRAC) with sleep every 2-4 hours without connections to the day-night cycle ([787, 372]).

- According to [1431] the sleep mechanism uses a functional unit of neurons in the central nervous system which is able to show rhythmic differences in spontaneous activity. The eye of Aplysia serves as a model. Using different control mechanisms in a diffuse system (*Formatio reticularis*) the thalamocortical system and the limbic system are connected. The same pacemaker neurons lead either to the wakefulness or the sleep stage. A rhythmic secretion of transmitter like substances of the nuclear metabolism affect the cell membrane and induce permeability- and excitatory changes.

[8] HVSA: high-voltage slow activity; LVFA: low-voltage fast activity

20.8 Melatonin

In the sixtieth of the twenties century it was found that melatonin serves photoperiodically reacting animals as a signal for the annual rhythm of reproduction: The pattern of the nocturnal melatonin production changes with the length of the night. Melatonin is an indicator of darkness and mediates the photoperiod to the organism.

Melatonin is a biologically old active molecule and phyllogenetically conserved. It is present already in the unicellular alga *Lingulodinium* ([47], [48], [50], [1168], [564]) and in *Pterygophora* ([464]). It has also be found in plants, insects, crustaceans, mollusks and worms ([1518]).

In the last decade of the twenties century it was discovered, that this indole

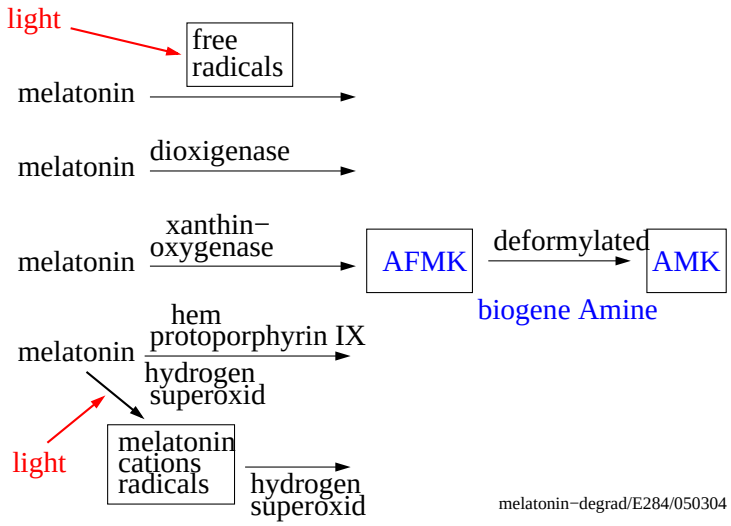

Figure 20.7: *Scheme of the degradation of melatonin by light, which has formed superoxid anions. They are converted by light with heme or indolamine 2,3-dioxygenase (IDO) to N1-acetyl-N2-formyl-5-methoxykynuramin (AFMK), which is transformed by arylamin-formamidase to N1-acetyl-5-methoxykynuramin (AMK). After [561]*

traps free hydroxyl radicals very effectively and acts anti-oxidizing. Free radicals are toxic and represent an oxidative stress to the cell. They are especially dangerous in the brain. Melatonin reduces this stress in different ways ([1219]). Melatonin furthermore stimulates gene expression of enzymes involved in antioxidant management. Superoxid anions, formed by light, are degraded to kynurenin (AFMK) by melatonin with hem as a catalysor (figure 20.7, [47], [564], [50]).

Melatonin is degraded in four different ways (see figure 20.7 and several articles in [562]) and excreted. Degradation is important for all hormones. Otherwise the target cells would not respond anymore to the hormone.

Melatonin is synthesized from tryptophane in four steps (figure 20.8, [214]). The structure of melatonin resembles that of auxin, gibberrelic acid, and cytokinin.

Melatonin occurs in all vertebrates. The main sites of synthesis are pinealocytes in the pineal gland and cells in the outer nuclear layer of the retina in the eyes. The pineal and melatonin convey the photoperiodic information in the environment to effector organs ([488]). This system regulates the seasonal timing of reproduction, hibernation, pelage color and other physiological processes ([213], [1532]). In rodents, for instance, melatonin suppresses all reproductive activity during the winter as a central strategy, thus helping to save energy. The specialties of melatonin and the pineal in mammal reproduction are treated in section 3.7.

Melatonin is not only involved in the regulation of photoreception and visual function ([149]), in photoperiodic reactions involving neuroendocrine events ([670]), but also in immune reactions ([305], [306], [946]).

It was tested, whether the immune system is also strengthened ([1065]). For this purpose

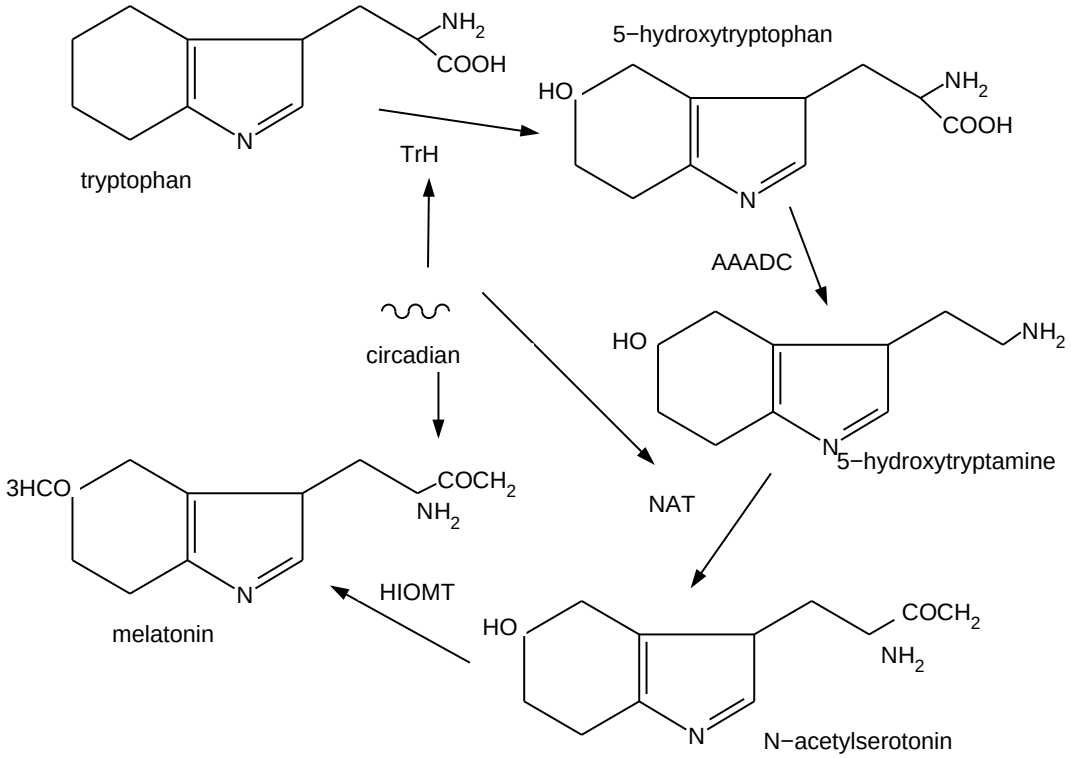

Figure 20.8: *Melatonin-biosynthesis: Tryptophane is transported via the blood to the pineal organ. In the pinealocytes it is hydroxylated by tryptophane hydroxylase to 5-hydroxytryptophane and decarboxylated by aromatic aminoacid decarboxylase to 5-hydroxytryptamine. With arylalkylamine N-acetyltransferase serotonin is formed. This is transformed by hydroxyindol-O-methyl transferase (HIOMT) to melatonin. The circadian clock controls three of the four enzymes involved at the transcriptional and post-transcriptional level. After [214]*

Peromyscus maniculatus were injected with a carcinogen DMBA. 90% of the animals kept under long day obtained cancer, but none of the animals kept under short day.

In vertebrates melatonin is secreted from the pineal in a circadian rhythm. Light during the night phase shifts this rhythm ([1367]). On the other hand, melatonin is an effective phase shifting agent of the circadian rhythms ([1205]).

Melatonin interferes furthermore with the body temperature control ([193]).

It has a homeostatic effect on core body temperature (via the POAH) and affects the circadian control of body temperature via the SCN. Melatonin secretion at night decreases body temperature. If melatonin is administered during the day (when it is not normally secreted), the body temperature decreases also, by about 0.3^0 to 0.4^0C. If melatonin secretion is suppressed at night the body temperature is increased by about the same magnitude. How melatonin decreases the body temperature is unclear. It might enhances heat loss, but could also reduce heat production. It is, however, more likely that it exerts its effect mainly in the hypothalamus, where thermoregulatory centers are located. The acute thermoregulatory effects of melatonin and of bright light are independent of their circadian phase-shifting effects. The effect of melatonin ultimately brings a saving of energy ([193]).

Melatonin has in addition a sleep-promoting effect and has been claimed to be a chronobiotic helping to correct circadian rhythm disorders in blind people, shift workers and in jet lag problems ([1297], [22]). Whether it is safe to apply melatonin externally is discussed ([129]).

It may have a modest direct hypnotic effect, but could also correct circadian phase abnormalities. In this case it might interact with melatonin receptors in the suprachiasmatic nucleus (SCN). As a result the circadian pacemaker could be reset and/or SCN-dependent circadian alerting processes could be attenuated. For details see [326], [1609], [998].

Finally, melatonin plays also a role in development. For the fetus it is a window to the external world telling it the time of year and the time of day ([296]). Via melatonin the mother is able to entrain the circadian system of the fetus. In hamsters photoperiodic effects can be transmitted to the fetus and determine its fate: Whether they will develop and reproduce or whether they will first hibernate before reproducing. Melatonin is also involved in puberty. Even in humans seasonal rhythms are conveyed by melatonin ([1538]). Melatonin secretion in humans is longer while sleeping in long nights (natural winter conditions) as compared to short nights (natural summer conditions, see upper part of figure 20.9. However, since most people in modern environments use the same daylength during summer and winter for their sleep pattern by the help of artificial illumination, these differences do not show up (see lower part of figure 20.9).

20.9 Synchronous menstruation among college students

Menstruation is a bleeding of the uterus which lasts 3-5 days. The uterus mucous membrane (endometrium) is discharged. The ovarian and uterine cycle lasts as an average 29.5 days. Since the moon needs 27 sideric days and 29.5 synodic days (the earth moves too), it was tempting to assume a relationship between the menstruation cycle and the moon cycle. In equatorial monkeys of south America such a correlation was indeed found. Menstruation occurs at the time of new moon, 14

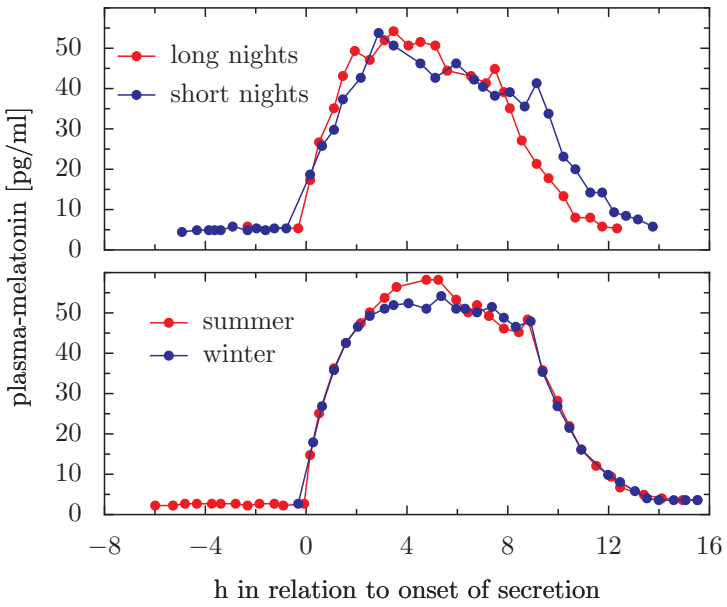

Figure 20.9: *Top curves: Plasma melatonin secretion in long (red) and short nights (blue). Bottom: Plasma melatonin secretion during the summer (red) and during the winter seasons (blue). After [1538]*

days later at full moon ovulation and conception take place. Whether this implies a selective advantage or is a social effect is unknown. In humans no fixed phase relationship between moon cycle and menstruation cycle was found ([1167]).[9]

There are, however, some indications for a synchronized menstruation cycle between woman.[10] Against this assumption speak, however, other observations ([1568], [694], [1487]).

A study has been performed at the Leibniz college in Tübingen (Germany), whether menstruation in woman can occur synchronously. Students living together in pairs in a double room and spend much time together were studied by [1356]. 19 of the participants of the study had a regular cycle, in 12 participants the cycles fluctuated more strongly. In three of the 13 cases menstruation between the students of one room was in synchrony. There was a tendency of a synchronized menstruation between several students ('phenomenon of the garbage containers filled with sanitary towels'). It would be interesting to repeat such a study with a larger number of women for a longer time. One could for instance deliver numbered sanitary towels and use the discharged one for daily notes.

20.10 Schizophrenia as nocturnalism

An ethological hypothesis was put forward, according to which the mental disease schizophrenia is a kind of nocturnalism ([419]). It is claimed that schizophrenia is a condition in which the brain is in its sleeping state during the day time. The hypothesis predicts improvement, if light is kept under a threshold value.

Schizophrenia is the most common mental disease ([826], [114]). 1% of the population shows it phenotypically. There is so far no way of treating it successfully. Schizophrenics are asocial and solitary. They have wrong (illogical) ideas ('delusions'), interpret sensory perceptions wrongly ('hallucinations), although they perceive correctly (short term memory is not affected). In their nonverbal behavior they show inappropriate affective behavior, the motoric pattern is funny, sometimes bizarre. Schizophrenics think unorderly, are not goal oriented, normal associations between ideas are disturbed ('tangential thinking').

Schizophrenia is determined by a genetic and a non–genetic component. So far there are no clear biochemical

[9] although there are some hints, that in certain cultures and native tribes such relations do exist. A woman of the Yurok Indians from California reports, that in earlier times all non-pregnant, fertile woman of a household menstruated at a certain time dictated by the moon ([1329], page 200). Further indications are available ([317], [316]).

[10] [975] has interviewed (1) 33 pairs of female student roommates, (2) 33 pairs of close female friends in separate rooms, (3) 33 pairs of close female friends in the same rooms and (4) 33 random pairs of female students. Group (2) (33 pairs of close female friends in separate rooms) menstruated synchronously. Likewise [522] found synchronous menstruation in 18 pairs being very close friends. [1190] and [1287] transferred axillary pheromones to the upper lip. By this they were able to shorten the average time of menstruations from 9.3 days after four months to 3.4 days. The experiments were repeated by [1177] using an alcohol control. The sensitivity toward the pheromones fluctuated with the menstruation cycle. [1546] found in lesbian women living together permanently a high synchronization of menstruation. Synchronization of menstruation was observed also between mother and daughter in the same house ([1547]). According to [316] the menstruation cycle can be regulated by light

Table 20.2: *Condition of the brain of normal (genetically diurnal) and schizophrenic (genetically nocturnal) persons during sleep and wakefulness in the light and in the dark. After [419]*

	diurnal		nocturnal	
	light	dark	light	dark
sleep	P	P	P	wP
wake	NP	NP	*P*	NP

and/or morphological correlates, by which schizophrenics and normal people can be distinguished.

Feierman proposes, that schizophrenia is not brought about by an inherited metabolic malfunction. Instead it is a phyllogenetic adaptation: The brain uses normally new informations of the environment during wakefulness and stores it in the short term memory of the limbic system. During sleep these stored informations are symbolized and categorized. Afterward they are stored during the P-state ('programming mode') in the long term memory (using cerebrospinal proteins?). Schizophrenics perceive informations of the environment with a brain in the P-state, if wake during light. From a genetic standpoint they are, however, nocturnal: During darkness they would be in the active non-P-condition, which normal people experience during the wake state in the light (and in the dark) (see table 20.2).

This means perhaps from a phyllogenetic view, that for a social animal living in a group such as man it is more favorable if a part of the group members is night-active (protecting the group, hunting during the night). A number of indications are in favor of this interpretation: Many schizophrenics are born during the winter (photoperiodic influence?). Among blind people no schizophrenics are found. Likewise, under narcoleptics with a polyphasic sleep pattern schizophrenics are absent. Many schizophrenics are more active during sleep as compared to the wake time. The muscle tonus is not suppressed during REM sleep. In the light or in weak continuous red light the symptoms of schizophrenia should disappear. Perhaps schizophrenics possess a special visual system in respect to the anatomy?

20.11 Lithium - experiments in Spitsbergen

Endogenous depressions are relatively frequent among humans (and in the meantime animal models are also available). This mental disease consists often of a manic and a depressive phase (bipolar depression). The manic phase might be absent (unipolar depression). During a depressive episode patients feel sad, hopeless, pessimistic, guilty. Energy and activity are reduced, concentration and memory impaired. Sleep is disturbed (more shallow, increased awakening, sleep efficiency reduced, sleep shortened by early awakening, REM latency advanced). Libido is decreased. Other activities in which normal persons take pleasure are not gratifying for the patients. They become often self-preoccupied and avoid social contacts. Mental anguish, hopelessness and self-blame lead sometimes to suicide.

The cause of this disease is not yet understood. There are some indications (as described in several articles in [1541] and [549]) that the circadian system seems to be altered in depression and might even be causally involved in this mental disorders. According to one hypothesis the phase of

a specific circadian rhythm is advanced in depressive patients. This could explain the observed shortened REM latencies, the advanced (earlier occurring) rhythm in body temperature, secretion of electrolytes, neurotransmitter and hormone levels. Several hypotheses have tried to link the disease to specific abnormalities in the circadian system such as free run of one circadian component, internal desynchronization among two or more circadian oscillators, changes in coupling of oscillators. A hypothesis which involves a photoperiodic reaction has also been proposed.

Among the different therapies used in endogenous depression treatment with Li^+ salts has shown to improve especially the manic phase. In view of the mentioned disturbances of the circadian system in depression it was interesting that Li^+salts have been shown to increase the period length in a number of plants and animals ([378]). If in depression a part of the circadian oscillatory system is too fast to be able to synchronize with the 24 hour Zeitgeber of the environment, they might free run or show a phase advanced relation to the other oscillators. The therapeutic effect of Li^+salts might consist of slowing this fast oscillating component thus normalizing the disturbed circadian system.

In order to test this proposal we tried to find out whether Li^+ salts lengthen also the circadian rhythm in humans, as was found before with the circadian rhythms in other organisms. We conducted an experiment in 1979 in Spitsbergen ([714]). This island was chosen because of its position high up in the north (79^0N) and therefore the sun never settles below the horizon during the summer. This allows to study the circadian rhythms of man under constant conditions, if the participants do not use any Zeitgeber of the 24-hour-day such as clocks, radio, TV. Four students from Norway and six students from Tübingen participated in this experiment. They lived in groups of two (for safety reasons) for four weeks in huts in the surrounding of Ny Alesund. Body temperature and arm movements (as a measure of the locomotor activity) were recorded automatically and the sleep time noted. Either in the first half or in the second half of the experiment lithium carbonate tablets were taken, and placebo-tablets during the control time.

During the time of taking lithium carbonate the periods were longer (figure 20.10). We concluded from this experiment, that Li^+ lengthens the period of the circadian rhythm of humans, as was found for other organisms (and monkeys also in the meantime, [1551]). Depressives might have (under normal conditions) a circadian rhythm (one out of others of the circadian system?) which is too fast and can therefore not be entrained to the 24 hour rhythm of the environment. By taking Li^+ this rhythm becomes lengthened in its period and can now entrain again to the 24 hour day. Alternatively Li^+ might change the coupling strength between circadian oscillators and the fast oscillator can be coupled again to the slower one, preventing it from free running ([389]).

20.12 Examples for bioluminescence

Lingulodinium polyedrum exhibits a circadian rhythm in bioluminescence (see chapter 4). Bioluminescence is found in numerous other organisms too. In all cases a luciferin Lf is converted by the enzyme luciferase Lfase and oxygen to oxidized luciferin OxLf. During the process light is

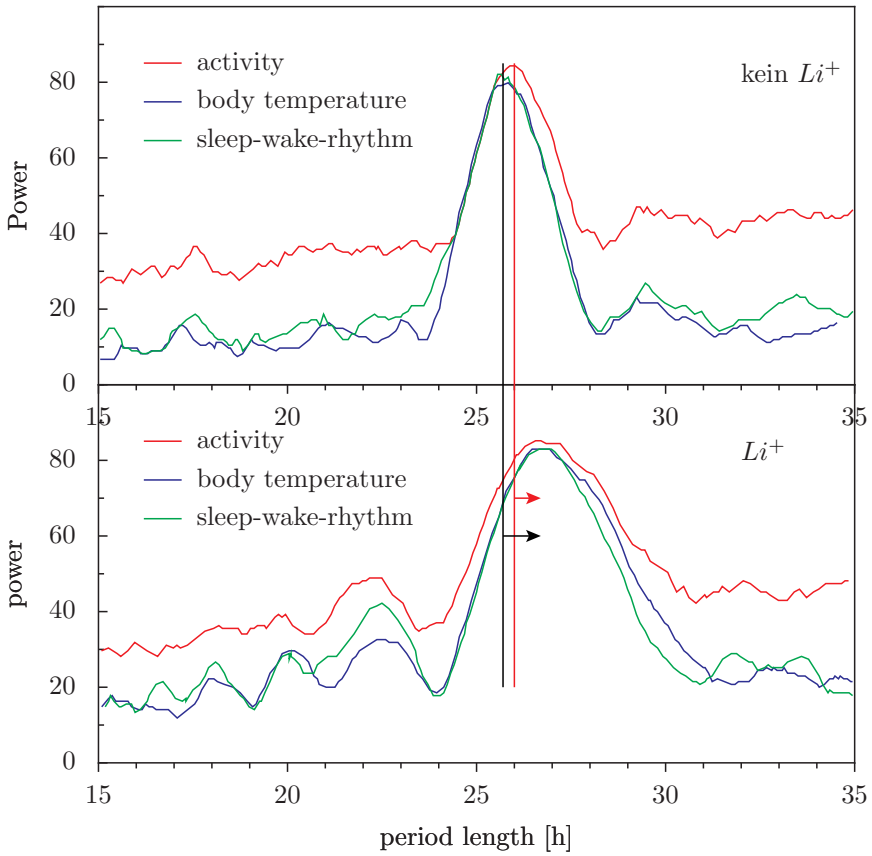

Figure 20.10: *Lithium salts lengthen circadian rhythm in humans: Periodogram of three different circadian rhythms in one of the subjects measuring activity (red), body temperature (green) and sleep-wake rhythm (blue) with placebo (top) and with* Li^+*tablets. All three peaks at 26 hours in top curve, all three peaks at 27 hours and peak of body temperature rhythm at 22.5 hrs are significant at the 95% confidence level. After [715]*

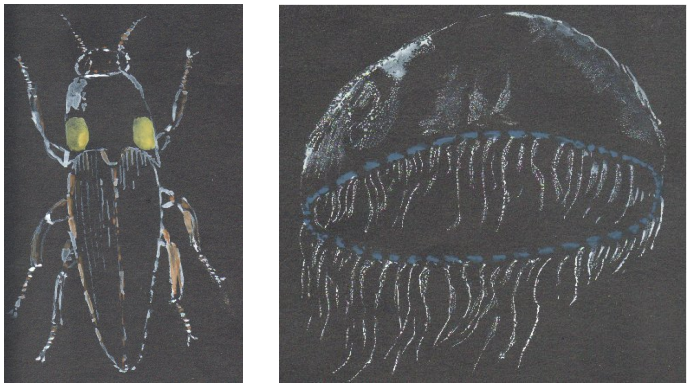

Figure 20.11: *Left: Bioluminescence of bacteria living in pockets of the thorax of an adult beetle. Right: Bioluminescence in a jellyfish. Drawn by the author after [978]*

produced. Lf is often heat stable, Lfase not. Figure 20.11 shows two examples.

Here are some other examples for bioluminescence:

Bacteria *Achromobacter fischeri* (=*Photobacterium phosphoreum)* uses a straight chain aldehyde R*CHO, which is converted by a LFase to R*COOH and light. In this case not a classical Lf is involved, but two cofactors. To measure the emitted light a photomultiplier is used in connection with a Peltier element and a thermoelement.

Fungi Bioluminescence is also found among a number of fungi. In some of them the light emittance is under circadian control: *Panus stipticus*, *Armillaria mellea* (), *Myceana polygramma* (). The chemical reactions are:

$(DPNH\,system + Lf \Rightarrow DPNH\,Oxase \Rightarrow Lf H_2 + DPN\ Lf H_2 \Rightarrow LFase, O_2 \Rightarrow Lf + H_2O + L$

Insects Among insects bioluminescence is present in *Photinus pyrralis* (*Lampyridae*, about 2000 species), *Lampyris noctiluca*, *Phaussis splendidula*, *Luciola noctiluca*, *Photuris*, *Platyura*, *Pyrophorus*, in the Elaterids *Phrixothrix* and in *Orfelia fultoni*. The larvae of *Arachnocampa luminosa* from New Zealand possess a light efficiency of 88% (562nm). They use the ATP-system

$Lf H_2 + ATP + E \Rightarrow Mg \Rightarrow E\,Lf H_2\,AMP + pH\,E\,Lf H_2\,AMP \Rightarrow Lfase, O_2 \Rightarrow Lf + H_2O + AMP + L$

The structure of the luciferin of this animal was the first among the luciferins to be clarified. It is completely different from other luciferases and is the only natural benzothiazol-derivative. It has been synthesized.

Crustaceae The Japanese Ostracode *Cypridina hilgendorfii* is an inhabitant of sandy coasts. During the night it is active at the water surface. Two glands at the upper lip show bioluminescence. The dried powder is used in Japan as a light source during the night. $Lf H_2 + O_2 \Rightarrow Lfase \Rightarrow Lf + L$

Hemichordata *Ptyclodera* (an enteropneust) contains photocytes.

Balanoglossum $2Lf H_2 + H_2O_2 \Rightarrow$

$Lfase \Rightarrow Lf + H_2O + L(peroxidase - system)$

Dinoflagellates Besides *Lingulodinium* other dinoflagellates exhibit also bioluminescence such as *Noctiluca*, *Pyrodinium bahamense*, *Gymnodinium breves*. They use a charged system:

$Lf \Rightarrow Lfase \Rightarrow Oxi\,Lf + L$

Coelenterates Bioluminescence in Coelenterates is found in *Renilla*.

$Lf + Lfase \Rightarrow O_2 \Rightarrow Oxi\,Lf + L$.

In the jellyfish *Aequorea* Luciferin is absent and O_2 is not used. However, Ca^{2+} is needed to excite the photoprotein. Aequorin is used for Ca^{2+} determination.

Significance of bioluminescence In a species bioluminescence might serve to recognize and find mates (glow worms), or for courtship (Polychaet *Odontosyllis*), the Palolo of the Atlantic, or for swarm formation, or marking of a territory.

Between species bioluminescence is used for the illumination of the view field for instance in muddy water. With the help of bioluminescence prey can be attracted (luminescent organs of the fish *Anomalops*, which are used in Japan as bait for fishing). Bioluminescence might serve also as a protective means by deterring enemies, as camouflage, as a method to distract enemies from the anterior end of the body as found in some worms.

Chemically bioluminescence can be used to get rid of H^+: It is a kind of short circuit for electrons of pyridine nucleotides. It is an effective method to reduce NAD^+ under O_2-shortage. Originally bioluminescence was probably a side effect of chemical reactions to expel O_2 from the system. Later, organisms could use O_2 directly in the metabolic machinery. Bioluminescence was not any more of advantage, was however under certain conditions still used, for instance in order to compete with other bacteria. O_2 deficiency induces luminescence. This can be used in some fish which contain luminescent bacteria to control their bioluminescence.

20.13 Photoreceptors for synchronization of circadian rhythms

Circadian rhythms have to be synchronized by environmental time cues to the 24-hour-day. Otherwise they would soon loose measure and would not serve any more as a clock. For synchronization photoreceptors are needed, which perceive light and send signals to the circadian clock. These photoreceptors are quite diverse, depending on the organism. They can be just simple pigments in a cell or highly developed eyes, to mention two extremes.

We will present three examples and look more closely into them:

1. The phytochrome system
2. The circadian controlled vision of the horseshoe crab *Limulus polyphemus*
3. The eye of mammals

More examples can be found under subsections 15 and on pages 326 and 311.

20.13.1 The phytochrome system

Light plays a paramount role in the development of plants. From seed germination, growth of the seedling to flower formation it governs important events and

Figure 20.12: *Seed germination of lettuce is induced by red light and inhibited by far red light. If red light (1 minute) and far red light illumination (4 minutes) follows each other, the last light color administered decides whether the seeds germinate or not. From left to right: R; R,FR; R,FR,R; R,FR,R,FR,R,FR,R,FR. In the case of multiple illuminations with red and far red the light which was given as the last one decides whether the seeds germinate or not (from [1303], after Borthwick)*

developmental steps. If for instance the seed of a light germinator is illuminated, almost all seeds will germinate. In darkness, however, no germination occurs (figure 20.12). Red light promotes this event, whereas darkness or far red light inhibits germination. If the seeds are illuminated first with red light and shortly afterward with far red light, they will not germinate. Decisive is the color of the last illumination.

Numerous events during development and morphogenesis of plants are controlled by the interplay of red and far red light. It is based on the phytochrome system (table 20.3). Phytochrome is also often involved in rhythmically controlled processes and in photoperiodic timing. Therefore we should go a little bit more into details.

Phytochrome is a blue pigment. Red light (maximal effect: 666 nm) converts it up to 85 % in an olive green form (with maximal absorption at 730 nm). This is the physiologically active form. Far red light reverts it back into the red absorbing form (figure 20.13). Reversibility is characteristic for phytochrome. The absorption- and action spectra are shown in figure 20.14. Maximal absorption and strongest effect are by the red (P_r) and the far red wavelengths (P_{fr}). In the blue absorption is minimal. Green light does not have an effect and can therefore be used as safe light. Illumination leads to a balance. It is described by $\Phi = \frac{P_{fr}}{P_{total}}$. Using red light the balance is $\Phi = 0.85$. That is, not all P_r is converted into P_r, because the absorption curves of the two pigment forms superimpose partly. In this way red light converts a part of the P_{fr} back to P_r.

The pigment was extracted and purified. It has been studied also in vitro. The reversibility occurs in vitro too, and as in vivo low light intensities are sufficient for conversion. The pigment is a tetrapyrrole (figure 20.13). It is similar to phycobilin of red algae and *Cyanobacteria*. It is bound via a sulfur-atom to the cystein-residue of a polypeptide. The polypeptide is a homodimer consisting of two 120 kDa polypeptides with 1200 amino acids each. Each polypeptide consists of two subunits of 74 and 55 kDa, which dimerize at the C-terminal end (figure 20.15). While absorbing red light the chromophor (the tetrapyrrole) is isomerized from the cis- into the trans-form. The structure of the polypep-

Table 20.3: *Control of different processes in plants by the phytochrome system. After [208] and [726]*

process	signal	information	effect
seed germination	sun light	surface of soil	VLFR
De-etiolation	first transition dark /light	grow out of soil	VLFR,HIR
early detection of neighbors	darkness /light	$\downarrow \frac{R}{FR}$ perceived by PhyB	?
avoid shadow	much light, little light (shadow)	neighbors, shadowing objects	HIR
set clock anew	light-on, light-off	duration of the natural day	VLFR,HIR
flowering	day length	season	HIR

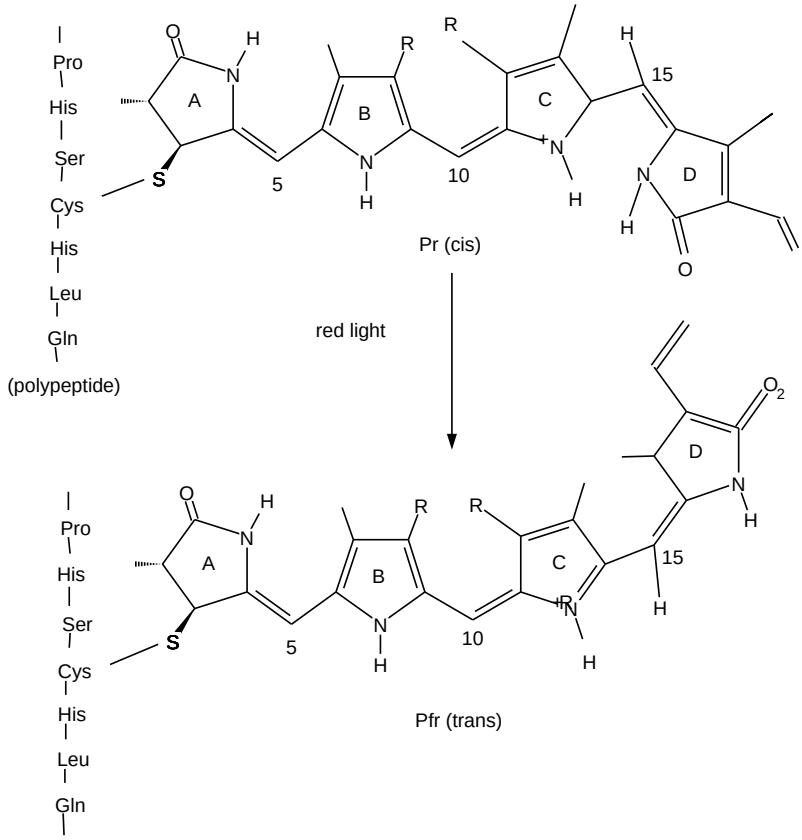

Figure 20.13: *Chemical structure of phytochrome chromophor, a tetrapyrrole, in its red- (P_r, cis) and far red- sensitive form (P_{fr}, trans)*

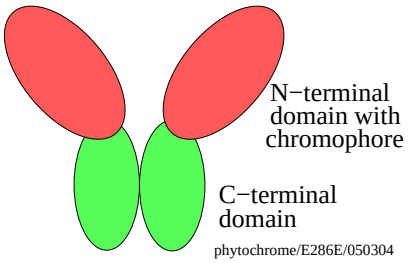

Figure 20.15: *Structure of holophytochrome. The molecule is a dimer consisting of two 120 kDa polypeptides (red and green) with 1200 amino acids each. Each polypeptide consists of two subunits of 74 (red) and 55 kDa (green), which dimerize at the C-terminal end. The phytochrome chromophor is a tetrapyrrole and attached to the N-terminal domain. After [1512]*

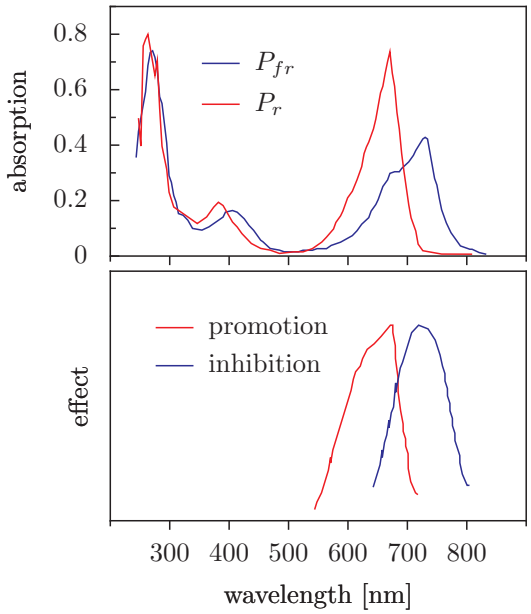

Figure 20.14: *Top: Absorption spectra for the red- (P_r) and far red-sensitive pigment system (P_{fr}) in* Avena *([1513]). Bottom: Action spectrum of phytochrome-controlled promotion of lettuce seed germination (red, P_r) or inhibition (blue, P_{fr}). After [134]*

tide is thereby changed in a way, which has not been clarified (polypeptide multiple configuration differences ([1512]). As a result, from the physiologically inactive P_r the physiologically active P_{fr} has formed. It is not known yet, how the different physiological effects are brought about.

A whole family of phytochromes exists which is coded by different genes. In *Arabidopsis thaliana* five such phytochromes and their genes are known so far (phytochrome A to E). They and/or their varying interactions are responsible for the different physiological effects. In darkness a young plant elongates. As soon as it is hit by light, this etiolation stops. Red light is the most effective, far red reverts it again. Phytochrome A is responsible for this de-etiolation of the seedling. But phytochrome B plays also a role (see figure 20.16). Phytochrome A and its mRNA are inactivated and metabolized quickly by light (see [237]). The other phytochromes (B to E in *Arabidopsis thaliana*) are stable.

How the different phytochromes

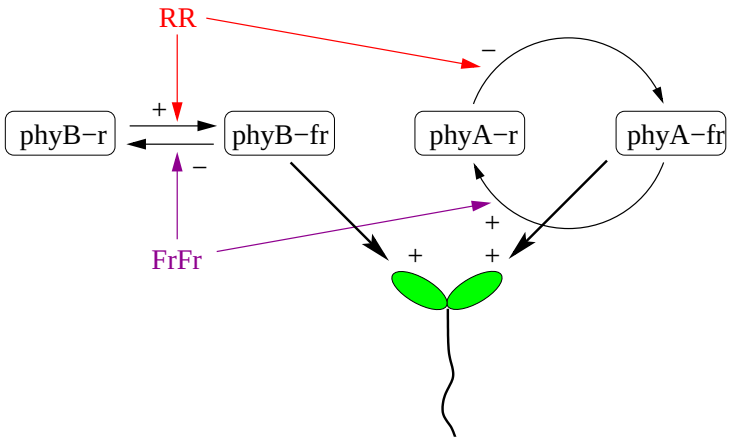

Figure 20.16: *Interaction of phytochrome B and phytochrome A in de-etiolating seedings: The red-absorbing form of phytochrome B (PhyB-r) is transformed by continuous red light (RR) in the far-red absorbing form (PhyB-fr). It stops etiolation (promotes de-etiolation: +). Phytochrome A (PhyA-r) is destructed by continuous red light (RR). Continuous far red light (FrFr) transforms the far-red absorbing form of phytochrome B (PhyB-fr) into the red-absorbing form (PhyB-r). In this way de-etiolation is prevented (the plants etiolate: +). After [1192]*

interact with each other and which further pigments are possibly involved in the phytochrome-reactions is studied presently.

Phytochrome can be determined and localized by spectral photometric or immunological means. The latter is by a factor of thousand more sensitive as the former. It can be used also in green tissue, whereas the spectral photometric determination of phytochrome is not possible because of the presence of chlorophyll. In this way phytochrome was established in cells. It occurs in the nucleus and in the cytosol, but not in the vacuole, the organelles and in membranes ([1529]).

Phytochrome was found in angiosperms, gymnosperms, liver mosses, mosses, ferns, some green-, red- and brown algae.

The synthesis of phytochromobilin from glutamate occurs via δ-aminolevulonic acid, protoporphyrin, hem and biliverdin in the plastid. Phytochromobilin is transported from the plastid into the cytoplasm (figure 20.17). There it forms together with the apophytochrome the holophytochrome ([1467]). To dismantle phytochrome ubiquitin is used. Interestingly the chlorophyll synthesis in plants and the hem synthesis in animals uses partly the same pathways. In mammals hem is metabolized via biliverdin and bilirubin to bilirubin-sugar-esters ([1113]).

Molecular biological and molecular genetic studies in the ninetieth of the last century have shed some light on the coding of the phytochrome system and its functions ([1191]) (see figure 20.18). The functions of the phytochromes can be divided into light perception (and also the interpretation of the light signals of the environment) and the regulatory functions (expression of genes for phytochrome-controlled reactions) by the activated phytochrome molecule ([1479]). There ex-

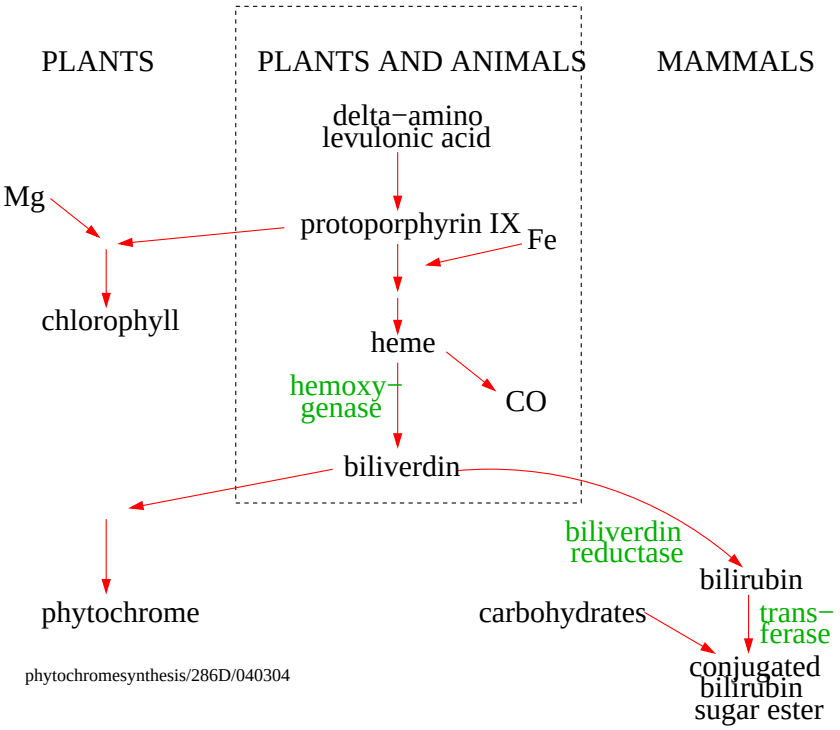

Figure 20.17: *Synthesis of phytochrome from δ-aminolevulonic acid, protoporphyrin, hem and biliverdin. The chlorophyll synthesis in plants and the hem synthesis in animals uses the same pathways up to the protoporphyrin. In mammals hem is metabolized via biliverdin and bilirubin to conjugated bilirubin-sugar-esters. In green some of the enzymes involved ([1113])*

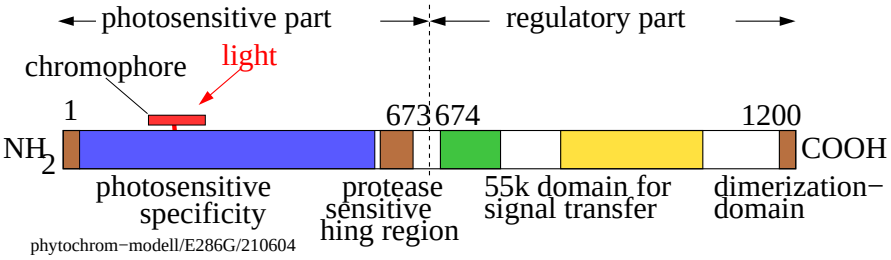

Figure 20.18: *Structure of the phytochrome-molecule (PhyA or PhyB) and the significance of different domains. The results are based on studies using mutants and deletions of transgenic plants. There are, for instance, mutants, which are normal in their photoreceptor-function, but defect in the regulatory function. Most of these mutations are found in a region of 160 amino acids (marked blue in the figure). The linear tetrapyrrole chromophor is indicated by the red part. The N-terminal 600 amino acid photo-sensory part determines the photo-sensory specificity of phytochrome. The green marked region (18 amino acids) is especially critical for the interactions between photoreceptor and signal transduction. It is separated from the photo-sensory domain by a protease-sensitive hinge region (black). The C-terminal part contains also the dimerization domain (yellow). At the N- and C- terminal are regions which affect the biological activity (brown). After [1191] and [65]*

ist furthermore attacking points for the destruction of the phytochrome molecule (proteolysis pathway via ubiquitin and proteasoms, [237]). More on it in figure 20.18. The light-sensitive functions of phytochromes are determined by the N-terminal part, the signal transduction onto consequent components by the C-terminal part of the gene. In the N-terminal part the position of the protein is coded, to which the tetrapyrrole-chromophor is covalently bound. The dimerization of the protein is determined in a region in the center of the gene and in the C-terminal part.

Which events occur after the activation of the phytochrome by light, in order to influence via intermediary processes the nuclear genome, is mainly unknown. Trimeric G-proteins, cGMP and Ca seem to be important in this connection. The signal pathways for the corresponding gene expressions are apparently pre-programmed and lead to the corresponding developmental programs. In the mutants cop, det and fus the seedlings develop in the dark as if they were kept in the light (that is, they do not etiolate). Apparently the products of these genes are involved in the light-dependent main switch (figure 20.19). This switch is situated between the input signals of the photoreceptor and the following cascades of gene expressions controlling photomorphogenesis. In the mutants hy5, fhy1 and fhy3, however, the seedlings etiolate in the dark in the same way as the wild type, but they do not react to light by de-etiolation ([139]). Here apparently the mutations have hit the machinery, which is responsible for the perception of light.

A two point contact-model of [1191] explains, how multiple sensoric events (wavelength, intensity, duration, periodicity) can lead to all the divers regulatory events such as seed germination, de-etiolation, avoidance of shadow, flower-

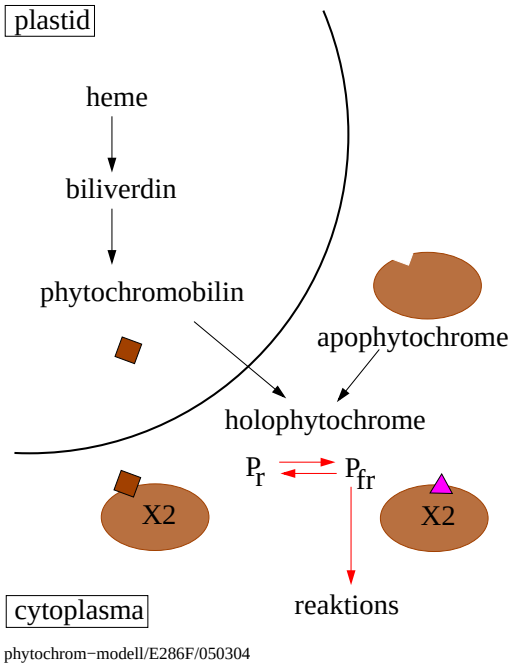

Figure 20.19: *Phytochrome A as a switch in morphogenetic events (etiolation/de-etiolation): P_r is the switch-off conformation, P_{fr} the switch-on conformation. After [208], [207]*

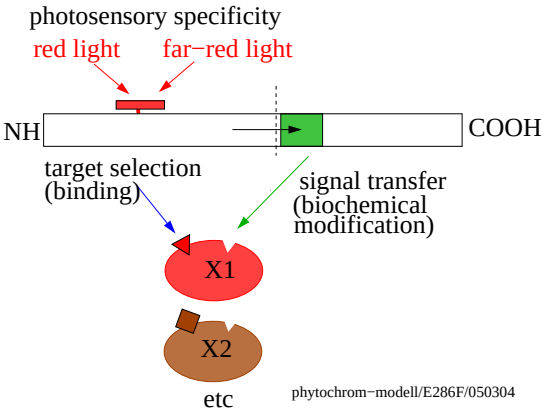

Figure 20.20: *A two point contact-model of phytochrome action: NH_2- terminal of phytochrome (B or A, red rectangle) contains specific recognition -determinants which bind (blue arrow) to separate reaction partners X_1 (red) or X_2 (brown) etc. according to the photo-sensory specificity (wavelength, intensity, duration, periodicity) (varying target selection). The light signal interacts (black arrow) with a core region (green) of the COOH-terminal. Biochemical modification by binding (green arrow) with a common determinant (indented triangle) which is present on all reaction partners X1, X2 etc. In this way different regulatory events such as seed germination, de-etiolation, avoidance of shadow, flowering can occur. After [1191]*

ing, as shown in figure (20.20). The pleiotropic control of growth and of development is usually under rules of gene expression.

The significance of phytochrome in photoperiodic reactions of plants has been discussed already in subsection 13.2.5. In the photoperiodic flower induction the light-stable phytochrome B inhibits flower induction and other photoperiodic events such as tuber formation. It is, however, not involved in the measurement of the day-length. The light-labile phytochrome A,

on the other hand, seems to be an essential part of this mechanism ([690]). In the longday plant *Arabidopsis thaliana* neither phytochrome A nor phytochrome B is responsible for the induction of flowering by far red presented at the end of the day. A further phytochrome seems to be responsible for it ([517]). Phytochrome C plays a role in the photoperiodic reaction of short-day plants, whereas phytochrome A has in this case no function.

20.13.2 Circadian controlled vision of the horseshoe crab

The horseshoe crab *Limulus polyphemus* (*Xiphosura*) belongs to the *Chelicerata*, a subphyllum of the arthropods. They are found at the Atlantic coast of north America from Yukatan to Nova Scotia. Circadian rhythms in the visual system are characteristic for them and quite similar to the one of scorpions ([432]) and orb-spiders ([1590], [1591]). In other invertebrates (crayfish, [21], [1123]) humoral processes are apparently involved. Limulus has lateral eyes, median ocelli and ventral photoreceptors. The central projections of the different photoreceptors have been visualized with the help of monoklonal antibodies ([196]).

In contrast to the situation in the marine snails *Aplysia* and *Bulla*, the retinas of the photoreceptors of *Chelicerata* (scorpions, horseshoe crabs, spiders) and *Crustacea* lack circadian pacemakers in the eyes. Instead, efferent fibers from the optic lobes of the brain send circadian signals to the visual structures of these organisms and modulate them. As a consequence, the lateral and median eyes show a well expressed circadian rhythm. As an example, in the scorpion *Androctonus australis* during the night the peak-to-peak amplitude of the ERG in response to brief flashes of light delivered every 30 minutes is five times more sensitive during the nighttime as compared to the daytime (see figure 20.21).

It is the rule, that sense organs do not only receive signals from the environment, code them and transfer them to the brain, but that they also receive signals from the brain. These central feedback signals prepare the organs to specific stimuli, adapt the sensoric functions to changes in the environment and/or control the metabolism in the receptors. Efferent neuronal paths or neurohormones can influence these feedbacks. The visual system of *Limulus* illustrates this well.

The circadian modulations of the visual system are brought about by a pair of circadian oscillators in the protocerebrum of the brain. The precise location of the circadian clock in the protocerebrum is not yet known. Anatomical and physiological results indicate that the medulla might be a candidate ([226], [367]). The two oscillators are coupled tightly by lateral neuronal connectives which synchronize the efferent activity of the two pacemaker ([55]). The synchrony is lost by cuts in the protocerebrum, but the activity remains. The cell bodies of the efferent fibers lie in the protocerebrum and seem to be neurosecretory. The cell bodies are either coupled with each other or receive synchronous inputs from the clock. The somata of the efferent fibers do probably not contain the oscillators. Rather, these (so far unknown) oscillators seem to form a neuronal circuit with the somata of the efferent fibers. The clock influences the sensitivity of the lateral eyes only in a certain time window during the night.

The efferent signal to the lateral eyes and the ventral photoreceptors consists of the simulta-

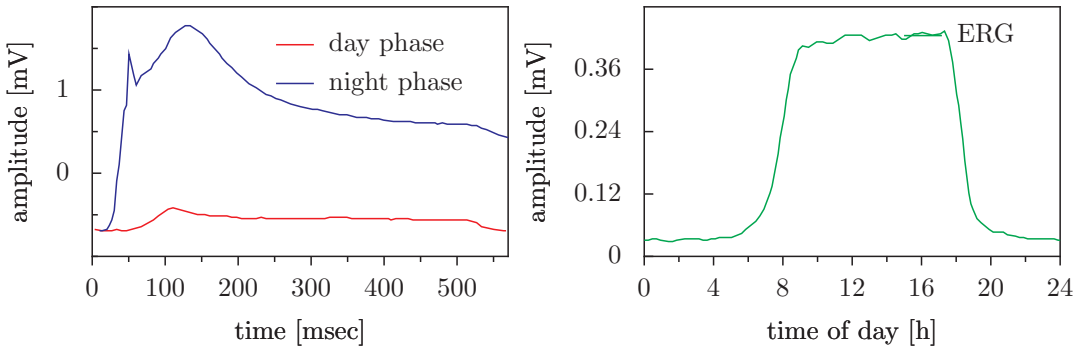

Figure 20.21: *ERG of dark-adapted median eye of the scorpion* Androctonus australis *(left) during dayphase (blue) and nightphase (red). The responses are induced by identical light pulses of 500 msec duration applied via a glass fiber. Reaction measured by a thin (20µm) platinum wire implanted laterally into the lens of the median eye. Indifferent platinum electrode ((100µm diameter) in cuticle rostral between the two median eyes. Signal amplified sent to oscillograph and tape recorder. Peak values of amplitudes show a well expressed circadian rhythm (right, green). The peak-to-peak amplitude of the ERG in response to brief flashes of light delivered every 30 minutes is five times more sensitive during the nighttime as compared to the daytime. After [431]*

neous firing of ten to twenty small fibers in the optic nerve ([417]). In the lateral eye the efferent fibers arborise heavily and terminate at retinular, eccentric and pigment cell bodies ([418]). In the ventral photoreceptor organ the efferent fibers terminate at the photoreceptor cells ([194]). For the median eyes no informations are available so far.

Under continuous darkness the circadian rhythm of the ERG-amplitude is preserved for at least a year. The period length during this time does not change significantly ([53]).[11] Seasonal and monthly variations in the sensitivity of the ventral eye were observed ([1269]).

Light pulses shift the rhythm to different amounts, depending on phase. The phase shifting light is probably received via the lateral eyes. However, the ERG rhythm can be phase shifted also by illuminating the *telson*. These phase shiftings lead to phase response curves which are normally obtained by dark pulses in other species with advances in the early subjective night and delays in late subjective night ([92], [1222]).

The circadian clock in the protocerebrum changes furthermore structure and function of the retina cells[12] in the lateral eyes. Shape of the photoreceptor- and pigment cells, pigment migration[13] and

[11] For individual animals the free run period differs and lies between 22.2 and 25.5 hours. The mean value is 23.9 ± 0.7h.

[12] There are large and small photoreceptor cells in the lateral eye of Limulus. Only the large cells show changes ([622])

[13] Pigment cells move away from each other during the night. As a result the diameter of the diaphragm increases. The retinular cells are closer to the base of the lens. The rhabdomer is 36% shorter and 34% broader. In cross sections the individual rays of the rhabdomer occur folded. In the longitudinal section they are folded.

photo-transduction[14] are affected ([56], [224]). The view field of the ommatidia increases[15] and the metabolism of the photoreceptors ([225]). This clock regulates also photo-mechanical movements within photoreceptors, the pigment distribution, the daily renewal of the photoreceptor membranes[16] via cytoskeleton-mechanisms and properties of the lateral inhibition ([64], [195]). All these effects increase the sensitivity of the visual system during the night. The underlying biochemical mechanisms are not well known yet[17].

These morphological changes occur also under continuous darkness. If the optic nerve is cut, the cyclic changes are gone and the ommatidia stay in the day state. If an electric pulse is applied to the stump, the night state is induced. Efferent fibers are therefore responsible for the structural changes. The view angle of the individual ommatidia changes from 6^0 during the day to 13^0 during the night. This is also inducible by electric stimulation: The activity of efferent optic nerves controls also the viewing angle. These and other changes allow dark adapted ommatidia to become 30 to 100 times more sensitive. The quantum yield is increased during the night, whereas the spacial resolution is decreased.

Single Limulus photoreceptors were recorded in vivo for several days and changes in their physiological and membrane properties studied. In vivo recordings at individual photoreceptors for several days show changes in the properties of their membranes caused by efferent signals from the brain. Probably ion channels in the membranes are influenced, thus increasing the sensitivity toward light ([743]). Electric stimulation induces the more sensitive night state. In this condition fast light changes are less well detected, but the sensitivity toward light is higher ([64]). In this way the circadian clock plays an important role in helping the animals to adjust to the light conditions of the surrounding.

The circadian clock increases also the sensitive for light of the median ocelli for wavelengths between 400 and 700 nm ([367]). The sensitivity toward ultraviolet light is, however, not changed, although these ocelli are most sensitive toward UV ([56]). Additionally, the circadian clock allows a photoreceptor organ to modulate the sensitivity of another one. This occurs

[14] The shape of quantum bumps change with the time of day, whereas the resting potential and resistance of the photoreceptor membrane do not change. The impulse rate of second order neurons, the eccentric cell, also remains unchanged with the time of day.

[15] The diaphragm formed by the distal pigment cells is tight during the day (17 μm diameter). The distance of the retinular cells to the lens is $30\mu m$. In this way the amount of light entering is limited. In a cross section, appendixes of proximate pigment cells with large granules are seen. They lie between neighboring retinular cells at the end of the rhabdomeric rays. Smaller pigment granules in the cytoplasm of the retinular cells are found close to the edge of the rhabdomers ([56]).

[16] So called membrane shedding. The rhabdomers are disintegrated with the first light of the day and renewed. The molecular mechanism by which light triggers the intracellular machinery responsible for the daily breakdown of the light sensitive membrane is under study. Rhodopsin absorbs the light through the phospholipase C/diacylglycerate/proteinkinaseC-cascade. It requires also elevated cytosolic Ca release by inositol triphosphate. The rhythmic shedding is a homeostatic mechanism to keep the quantum catch constant. Shedding is absent in animals in the deep see where no cyclic light occurs ([223]).

[17] A rise in cAMP concentration in photoreceptors and phosphorylation of a protein specific for the visual system (myosin III) is probably involved in some of the changes controlled by the clock ([67]). Cytoskeletal mechanisms participate in the control of the aperture and rhabdom shape, pigment movement and shedding of rhabdom membrane ([195])

only during the night, not during the day ([56]).

Even isolated photoreceptors (the large type) of the ventral eye change their form in the dark and in the light. In the dark-adapted state the microvilli are small and positioned in an orderly way (sometimes crystal like). In the light-adapted state, however, they are much thicker and unordered. The transition from one state to the other occurs rapidly and is completed after 30 minutes. This shows that the synthesizing as well as the degenerative phase of the renewal of rhabdoms can occur also in isolated rhabdoms. No efferent activity is needed. Light and darkness are already sufficient.

What is the significance of the circadian control of visual sensitivity in *Limulus*? The visual senses are not used much in the daily life of *Limulus*. However, they are important in finding mates. During the mating season male *Limulus* are attracted visually by females or by dummies. They recognize objects during the night almost as well as during the day. The eye adapts to the changed light conditions and can thus compensate the huge differences ([57], [1175], [624], [627]).

20.13.3 The eye of mammals sees besides images also the time structure of the environment

The eye of mammals does not only serve to see the visual environment, but also, to convey its time structure to the brain. In the paired SCN of the hypothalamus are cells which present the center of the circadian clock.

The circadian clock of vertebrates is synchronized to the 24 hour structure of the environment mainly by the light-dark-cycle, as is the case in other organisms. Whereas in non-mammals besides the eyes other light receptors are used for it, in mammals only the eyes seem to keep the circadian system in a daily measure.

Recently, however, a paper was published ([197]), in which an extraretinal synchronisation of the circadian rhythm was claimed. The hollows of the knee of volunteers was illuminated at proper times with bright light of 13000 lux. This shifted the rhythm of the body temperature. As photoreceptors the authors suspected heme-compounds such as hemoglobin or bilirubin (see also [1113]). Activation of these compounds by light could set free signal gases such as CO and NO ('humoral light transmission'). Other studies had shown that NO shifted the phase of the circadian rhythm in the SCN.

It is, however, doubted whether these findings are substantial ([448]). There are objections in regard to the experimental procedure. Furthermore it was pointed out that humans which lost their sight or who were blind from birth onward, lack circadian reactions to light ([279]). Numerous experiments with rodents show the same. Thus [1067] kept golden mantled ground squirrels without eyes under natural conditions. In contrast to the sighted controls the blind animals were not synchronized by the strong light (average intensity 55000 lux). Instead, the daily rhythm free ran. Weaker light intensities did not synchronize the circadian rhythm of the blind rodents either ([450]). Furthermore the action spectrum of phase shifting light corresponds to the absorption spectrum of opsin-photopigments of vertebrats ([1184]). This speaks in favor of the same basic structure of the photo pigments for synchronization of circadian rhythms on the one hand and visual pig-

ments on the other hand (namely opsin and retinaldehyde (= vitamin A) as chromophors). If tetrapyrrols were responsible for the synchronization, the action spectrum should show several peaks. This is, however, not the case.

On the other hand the rods of the retina are not needed for synchronizing circadian rhythms. The circadian rhythm of homozygous mice mutants rd/rd (retinal degeneration) can still be synchronized by light, although almost all rods of these animals with an age of 60 days are degenerated. In an age of 90 to 150 days the electrophysiological and behavioral reactions to coarse visual stimuli are completely absent. Only a few cones are left in this age, and they do not possess any outer segments. In spite of this the animals are still synchronizable. The threshold of the synchronizing light is the same as in intact control animals ([450]). Presumably the few cones without outer segments are sufficient for synchronisation. Transgenic mice without M-cones and with a few S-cones only are likewise synchronizable. Green light is sufficient for synchronisation, a wavelength, in which the rods are maximally sensitive. One could therefore assume, that the synchronizimg inputs into the clock are redundant. Rods as well as cones are sufficent for synchronization. Alternativly a so far unknown green-sensitive photoreceptor could be responsible ([1249]). Unknown retinal photopigments were found in fish ([1402]) and amphibia ([1183]).

The eye of mammals has thus two distinct sensory tasks: Image-forming and non-image-forming light detection. These two abilities of the eye are later parted in the central nervous system. In favor of it speaks:

1. There exists a population of retinal ganglion cells, which project to the SCN, but not to the visual centers of the brain ([1183]).

2. Blind moles (*Spalax ehrenbergi*) are able to synchronize their circadian rhythm, although their remnants of eyes do not perceive images anymore. The brain centers for image processing are lacking or are extremly reduced. In contrats, the SCN is well developed and receives projections of the remains of the retina ([248]).

20.14 Chloroplasts: Migration, changes in shape and pigment alterations

As in animals, plants are also faced with the problem of high light intensities at certain times of the day, the year or the particular situation (for instance exposure during low tides in marine algae). They had to develop counter-measures during the evolution which are effective enough to avoid damages in the photosynthetic system. A few examples are given in the following.

In several algae chloroplasts have been observed to migrate or undergo changes in shape and pigment concentrations ([1016]). These events can be under the control of a circadian rhythm as was shown in the case of chloroplast migration in *Ulva* ([156]) and in *Acetabularia* ([815]), in cases of changes in chloroplast shape ([1501]) and in the molecular structure ([1505]).

Halimeda has been found particularly suitable for studies of long-distance chloroplast migration by virtue of its

coenocytic structure and calcium carbonate skeleton. A circadian rhythm of chloroplast migration in *Halimeda distorta* was monitored by videography of segment surface pigmentation ([334]). In normal 12 h light/12 h dark treatments synchronized with dawn and dusk, the segments were green all day, began to become pale immediately the light was turned off, and then remained almost white for most of the night until beginning to re-green a few hours before dawn. As a result of that, they were already quite green by the time the light was turned on. In continuous darkness a similar cycle, albeit with reducing amplitude and a period of about 23 hours, was maintained for at least 7 days. However, this cycle differed significantly from the normal one in that the segments did not remain green after the light was not switched on at dawn, but rather began to pale immediately thereafter. Conversely, in continuous light the segments did not become pale at any time. Thus, the rhythmical re-emergence of the chloroplasts before dawn and their subsequent withdrawal appears to be controlled by an endogenous rhythm which is independent of light. However, light does completely, but reversibly, inhibit the chloroplast withdrawal component of the cycle. This behavior of the chloroplasts in *Halimeda* is very similar to that in the related alga *Caulerpa* ([586]), but it is quite different from that in another extensively studied but unrelated siphorous green alga, *Acetabularia*, in which the circadian rhythm of chloroplast migration is maintained in continuous light ([815]).

In *Dictyota dichotoma*, a brown alga, chromatophors are displaced from the periclinal (face position) to the anticlinal walls of the thallus cell (profile position) at high light intensities. This change in the position of the phaeoplasts can be observed under light-dark cycles and under continuous light or continuous darkness ([1097], figure 20.22). The rhythm is observed also in the *isolated* upper or lower cortical cell layer (a thallus of *Dictyota dichotoma* consists of three cell layers only, an inner colorless medullary layer and an upper and lower pigmented cortical cell layer). It was suggested, that in this way the algae are protected from high intensity light damage during low tides.

For references in higher plants see [587], for ferns [189]. An early account is [1364] and [1365].

20.15 History of photoperiodism

The day-length changes especially in areas further away from the equator a lot. This has enormous effects on climate, environment and organisms. Man is affected by it too. They have therefore been concerned with it from long ago. Winter- and summer solstice were celebrated in rites. One of the first scientific achievements of mankind was the calculation and prediction of seasons. The day-length plays a decisive role in these computations. Our calendar is based on it.

The significance of the day-length for organisms was, however, discovered relatively late at the begin of the 19th century, although 'Yogai´ singing birds were brought to premature gonadal development in the ancient Japan by using artificial longday and thus to singing. In the following a short overview of the history of the photoperiodism is given ([411], [220]):

- [614] assumed, that day-length as a function of the geographical latitude

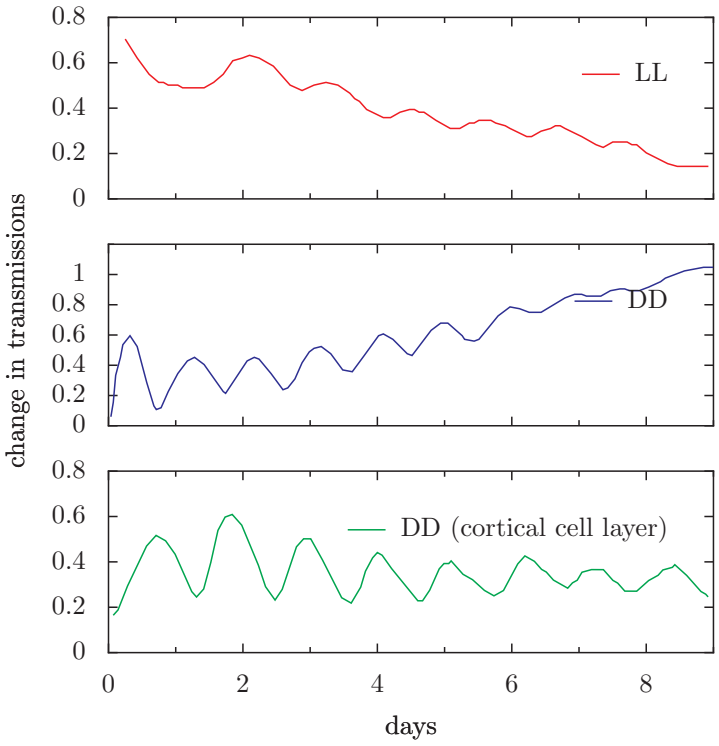

Figure 20.22: *Circadian changes in transmittance in* Dictyota dichotoma *thallus. The transmittance of light was measured by using a microphotometer with light of* $10^{-4}Wm^{-2}$ *at a wavelength of 439 nm. It was shown that transmittance is a measure of the position of the chromatophors being more frequently at the anticlinal or periclinal walls. The upper curve (red) shows the rhythm in continuous light, the curve in the middle (blue) at physiological darkness (weak blue light at* $10^{-4}Wm^{-2}$*) and the curve at the bottom the rhythm (green) of an isolated cortical cell layer. After [1097]*

is of significance for the natural propagation of plants and for their development.

- [781] showed in experiments at the polar circle, that plants develop faster under long day. He did, however, not exclude the photosynthesis as the reason for it.

- [40] used 'electrohorticultur' for studies and showed, that certain plants flowered premature, if the day was expanded by electrical lamps artificially. The invention of electrical bulbs by Edison in 1879 was the basis for these experiments.

- Turnois ([1483], [1484]) induced flowering in *Humulus* and *Cannabis* in short day. 1911 he found a premature flowering in plants sown during the winter. He showed, that neither temperature nor humidity or the origin of the seeds were responsible for it. 1912 he experimented with natural day light, continuous light (incandescence bulbs) and 6 hour light periods. Under 6 hours light periods the plants grew slowest, but flowered earliest. He considered the day-length as a factor, but concludes that the amount of light is the cause. 1913 he shows, that light intensity is without much influence, but that short day (=long night!) was responsible for it. He planned further experiments, but died shortly after the publication of his work in the first world war at the front.

- [783] studied at the same time the rosetta formation in *Sempervivum funkii*. He used incandescence bulbs and showed, that plants during the winter can be induced to flower by long day. Light was in this case a 'catalytic', not a nutrient factor.

- Garner and Allard discovered between 1918 and 1920 the 'winter factor' in a giant variety ('Maryland Mammoth') of tobacco ([478]). The plants grew 3-5 m high, but stayed vegetative even during the summer. In the winter the flowered already at a heights of 1 m. In searching for the 'winter factor' all kinds of hypotheses were put forward such as for instance the intensity and composition of the light. The mutant 'Maryland Mammoth' was than exposed in a kind of 'primitive dog hut according to own design' exposed to a day-length of 7 hours only. They were in this way induced to flower. This showed, that the day-length was the decisive factor in inducing flowering. It was furthermore shown, that soy bean plants came to flower around the same time of the year independent of the time of sawing. Radish, carrots, salad, winter barley reacted with flowering to the length of the day. The authors proposed for this finding the term 'photoperiodism'. The very readable publication was almost rejected by the journal, since it -as claimed- did not contain enough new findings.

- [792] studied the vernalisation of grain. Winter wheat flowers only after having been exposed for some time to lower temperatures. The studies were later continued by [480] using Petkus-rye (winter rye). [950] and [940] stress, that reproductive development and growth are different processes (which had been found quite early by [942]). [526] continued these

studies.

- [1554] discovers thermoperiodism.

Photoperiodism was likewise discovered as a main factor for the seasonal control of animal behavior:

- [1326] points to the significance of the day-length for bird migration.
- [956] studies the seasonal dimorphism in aphidsand supplies experimental results for a photoperiodic reaction.
- [1272] finds photoperiodic control of migratory behavior and gonadal maturation in birds (*Junco hyemalis*).
- [800] discovers insect diapause in *Bombyx*, a short day animal.
- [1294] finds in the same year photoperiodic control in grasshoppers.
- [43] observes in mosquitoes photoperiodic termination of diapause.

Thus a number of phenomena regarding photoperiodism and thermoperiodism were described. More were added in the following years. At the same time the search for the physiological basis of photoperiodic reactions began. In the induction of flowering by vernalisation it was shown that the tissue in the apex was responsible ([274]). The formation of tubers in potatoes ([1204]) and flower induction (*Cosmos*, [477]) is, however, induced by the leaves. Apparently under suitable photoperiods a substance is formed, which induces flowering photoperiodically ([798]). Sachs asked for a flowering hormone already in 1865 as the basis for photoperiodic induction ([1295]). After Went discovered auxin as a plant hormone ([1553]), the search for a flower hormone was intensified. [218] proposed the name *'florigen'* and later *'anthesin'* ([221]). He, Moshkov and Psarev ([219], [1039], [1185]) showed, that the leaves were the organs were these hormones are produced before being transported to the apex and to induce the apex to form a flower. Using grafting experiments the hypothesis was further tested ([835], [219]). The photoperiodic stimulus for flowering of the day neutral sun flower plant *Helianthus annuus* could be transferred to the short day plant *Helianthus tuberosus* (artichoke). The shortday plant *Nicotiana tabacum* Maryland Mammoth could be induced to flower by grafting the long day plant *Nicotiana tabacum* onto it ([1040]). Later experiments showed, that short- and long day plants use the same flower hormone.

Further important stations on the way of photoperiodic research were studies by [899] (florigen acid), [525], [555] (role of the dark period, light breaks in the middle of the dark period convey a short day to a long day), [310] (no flowering hormone, but removal of inhibition to flower), [917] (grafting). The light- and dark processes of flower induction were studied next and how they influence each other.

All these experiments were concerned with the photoperiodically controlled reactions and their mechanisms. Two further questions were important:

1. Which light is photoperiodically effective and which pigments receive it?
2. How is day-length measured?

In numerous photomorphogenetical processes the red and far red light plays an important role. The underlying pigment system was termed phytochrome ([190]).

It is important in the etiolation, seed germination and flower induction. Its chemical structure was classified ([1377]). In the meantime it turned out that a number of different phytochromes exist with different properties (Subsection 20.13.1).

The circadian rhythmicity, discovered already in 1729 by [299], was proposed to be the basis of the timing mechanism in photoperiodic reactions according to [180]: 'Photoperiodism had to be discovered in order to find a selective advantage for circadian rhythms'. [201], [992] and numerous other plant physiologists studied the interrelations. Later [1162], Hamner and Bonner ([555]) and others worked on problems of photoperiodic timing. How short- and long day plants measure time for the photoperiodic reactions was discussed first quite generally, later more detailed in models. The Bünning hypothesis and newer ideas and models are presented in the next section.

20.16 Models of the photoperiodic control

Photoperiodic reactions are widespread among organisms and examples were discussed before (see chapter 13). How day-length leads ultimately to a photoperiodic reaction has been extensively studied experimentally. Being a complicated issue, models have also been proposed which aim to describe formally the underlying principles and mechanisms.

One of the most intriguing questions is, how the photoperiodic information of the environment (that is day-length or night length or both) leads to the photoperiodic reaction (for instance flower induction). In the following some models are presented and discussed:

- The hourglass model.
- The Bünning-hypothesis, according to which circadian rhythms are responsible for photoperiodic time measurement. The following critical experiments and results speak in favor of the Bünning-hypothesis. But there are also results which are not in accordance with the hypothesis or demand modifications.
- The model of external coincidence.
- feedback models.
- The model of internal coincidence.
- The resonance model.
- Amplitude models.
- The photoperiodic counter model of Saunders and Lewis.

20.16.1 Hourglass model

An hour glass was used in ancient times to determine a certain time period, e.g. an hour for a ship watch or 4 minutes for boiling an egg. It consists of two glass bulbs connected with each other by a bottle neck. One bulb is filled with fine sand. If turned, the sand will run through the bottleneck which takes a certain time, depending on the diameter of the bottle neck and the amount of sand.

If an organism measures the length of a dark period it could do so with an hourglass mechanism. With onset of darkness a process could be initiated, which is terminated at the end of the dark period (onset of light period). Let us suppose that during this process a substance is produced, than the amount of it would be proportional to the length of the dark period. If

the concentration reaches a certain threshold (let´s say after 12.5 hours) it might in a particular case be able to induce the photoperiodic reaction (probably via a chain of other processes).

The photoperiodic reaction would thus be induced by all dark periods exceeding the threshold. Dark periods shorter than this *critical dark period* would be ineffective. An example is shown in figure 20.23 where the photoperiodic induction of morphs formation in *Megoura* is illustrated ([872]).

If a rather long dark period is offered, and groups of aphids are illuminated at certain times in the dark for an hour, the dark process would be terminated by the light pulse. If the light came too early, the critical dark period would not yet have been reached. The photoperiodic reaction would not occur. However, all light pulses occurring after this critical time would not prevent the photoperiodic reaction (lower part of figure 20.23). This is an experiment which could be used to test whether an hourglass is involved.

However, such an experiment might indicate, that an hourglass mechanism is involved, although this could be due to special conditions only. For instance, diapause in the flesh fly *Sarcophaga* can be induced by light-dark-cycles consisting of a 12 hour light period and a sufficiently long dark period. At a temperature of 16°C diapause is induced at all dark periods exceeding a critical length. However, using higher temperatures, the control by a circadian clock becomes obvious: depending on the length of the dark period, the percentage of diapausing animals is higher or lower (figure 20.24). Apparently, low temperature increases the percentage of diapausing animals to such an extent, that the clock influence is not visible any more.

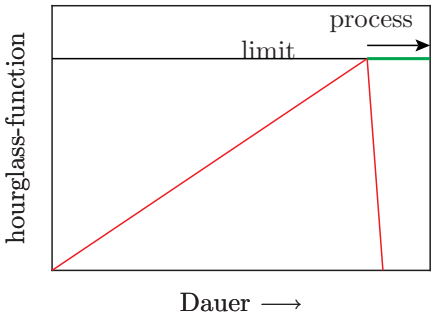

Figure 20.23: *Hourglass model of photoperiodic time measurement: With onset of darkness an hourglass function begins (red line) which increases with duration of the dark period. If long enough to reach a threshold an event is started (green line) which leads to a photoperiodic reaction. At the end of the dark period the hourglass function is reset and may start again with the onset of a new dark period. After [1225]; see also experimental data of [872]*

20.16.2 Bünning-hypothesis, external coincidence model

[180] proposed that time measurement in photoperiodic reactions uses the circadian clock. His view of flower induction is explained in figure 20.25. Light has accordingly two functions: It synchronizes the circadian clock, and depending on the photoperiodic situation of the season (long days or short days) and depending on the photoperiodic situation of the organism (for instance a long day plant or a short day plant) it induces the photoperiodic reaction or not.

The internal oscillation with its different circadian phases (photophilic and skotophilic) coincides with the external rhythm of the light-dark-cycle in different ways, depending on the length of the day. How the external light-dark cycle synchro-

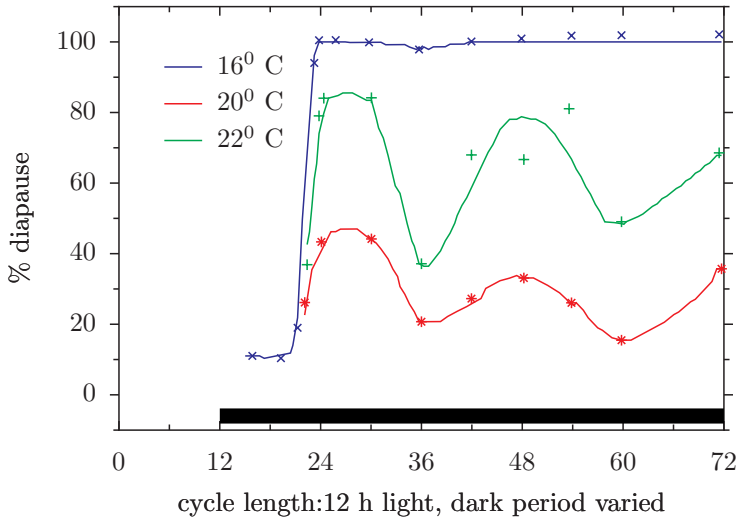

Figure 20.24: *Diapause induction in* Sarcophaga *(flesh flies) seems to follow an hourglass mechanism at* 16^0C*, but data from* 20^0C *and* 22^0C *show, that a circadian mechanism is involved in the determination of diapause. After [1316]*

nizes the internal clock can be different, depending on whether the onset of light or the end of the light period have stronger phase setting capabilities. In the example of figure 20.25 it is the onset of the light period which determines phase in both, the long-day and the short-day situation. However, it is more realistic, that both, lights on and lights off, play a role in phase setting. This will be explored in the next subsection.

Bünnings hypothesis has been modified by assuming, that a rather short part of the oscillation is sensitive to light (so called light inducible phase Φ_i) and that the external light-dark cycle has to coincide with Φ_i in the right way (see figure 20.26 and [1159]). This model has been called *external coincidence* in contrast to the *internal coincidence model* (see subsection 20.16.3). There is, however, no fundamental difference between the two models: The external light-dark cycle might well induce a rhythm correlate inside the organism, which than coincides or not with the critical internal oscillator phases. This is the situation in the feedback model discussed in subsection 20.16.4.

In a number of experiments the Bünning-hypothesis has been tested. [992] for instance kept *Kalanchoe* plants in 72 hour cycles consisting of a light period of 10 hours and a dark period of 62 hours. Different groups of these plants were illuminated with a one hour light pulse at various times of the long dark period and the average number of flowers determined. The flower induction varied as a function of the application time of the light break during the dark period, which indicates the control of photoperiodic induction by a circadian clock (figure 20.27).

20.16.3 Internal coincidence

Experiments with petal movements of *Kalanchoe* ([374]) led us to propose a model

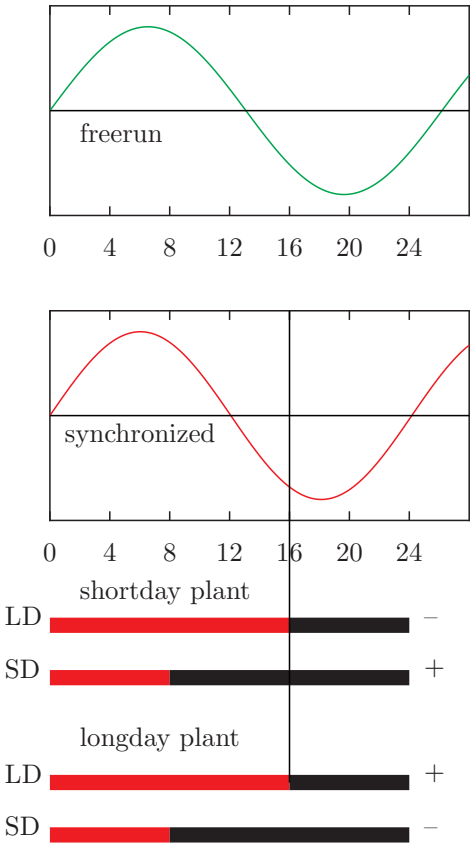

Figure 20.25: *Bünning model of the photoperiodic induction of flowering (or other processes). Light has two functions: First, it synchronizes the circadian clock to the light-dark cycle. The top curve shows a free running oscillation under constant conditions without Zeitgeber (light-dark-cycle or temperature cycle). The two curves below are entrained by light-dark-cycles (long day, center, short day, bottom) to the 24 hour day. Secondly, light affects the photoperiodic system differently, depending on whether short day or long day prevails. In the long day example the long light period (white space at top of x-axis) coincides not only with the so called 'photophilic phase´ (light-liking, red part of curve), but partly also with the skotophilic phase ('dark-liking´, gray part of curve). This induces flowering in a long-day plant, but prevents flowering in a short-day plant. Under short days the skotophilic phase is not illuminated and a long day plant will not flower. However, short day plants would be induced to flower. After [185]*

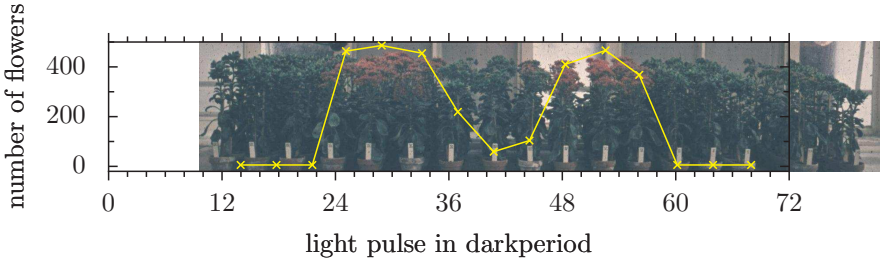

Figure 20.27: *Test of Bünning hypothesis by Melchers:* Kalanchoe *plants kept in 72 hour cycles of 10 hours light and 62 hours darkness. Average number of flowers per plant in different groups illuminated with one hour light pulse during dark period varies rhythmically, indicating the control of photoperiodic induction by a circadian clock. After [992]*

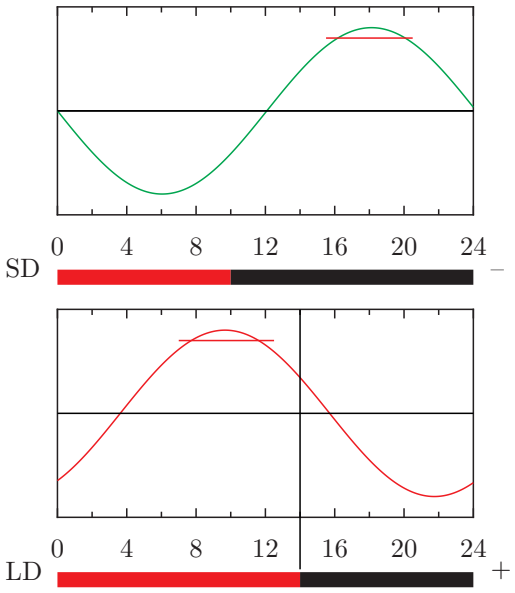

Figure 20.26: *External coincidence model for photoperiodic reactions. Light synchronizes the circadian clock to the prevailing light-dark cycle. Note that the phase of the oscillation is not fixed to dawn. Top curve (red): Under 14:10 hour long days. Bottom curve (blue): Under 3:21 hour short days. A light sensitive phase Φ_i of the oscillator above a threshold has to coincide with light in order to leads to a photoperiodic reaction. After [1159]*

for photoperiodic time measurement in which an internal rhythm is induced in an organism by the onset of light and an other internal rhythm by the onset of darkness ([375], [376]). The super-position of the two rhythms increases or decreases the amplitude of the resulting oscillation. High amplitude oscillations allow photoperiodic reactions to occur. To exemplify this internal coincidence model, the *Kalanchoe* flower induction is used in the following and the petal movement rhythm serves as hands of the two different oscillators.

If flowering *Kalanchoe* plants are kept for more than 14 days in continuous darkness (which is possible in this crassulacean plant; green as physiological darkness facilitates handling and watering of the plants), the flowers do not show movements anymore and are maximally open. Transfer of these flowers into continuous light conditions starts a circadian oscillation in the petal movement, which we termed *'lights on rhythm'* (figure 20.28). If flowering *Kalanchoe* plants are kept for a few days in continuous light, the flowers stop moving and are almost completely closed. Transfer of these flowers into continuous darkness conditions starts a cir-

cadian oscillation in the petal movement, which we termed *'lights off rhythm'* (figure 20.28). The figure shows that the first maximum of the lights on rhythm occurs 5 hours after the transfer into light, and the first maximum of the lights off rhythm is found 15 hours after the transfer into darkness. If a light period of 10 hours is offered to the flowers, the two rhythms coincide with each other in such a way, that the first lights off maximum and the first lights on maximum occur at the same time. This is surprisingly the critical day-length of the photoperiodic induction of flowering in *Kalanchoe.* We therefore proposed, that the kind of super-position of a *'lights on rhythm'* and a *'lights off rhythm'* is responsible for the photoperiodic reaction (here: flower induction) to occur or not.

20.16.4 Feedback models and photoperiodism

The main point of Bünning's idea of circadian clocks being used for photoperiodic reactions is that an internal oscillator in an organism is (1) entrained by the external light-dark-cycle and (2) depending on whether the skotophilic phase falls into darkness or is illuminated partly a chain of events is induced or not, leading to a photoperiodic reaction. There are however several points which are not clear enough and which have partly been addressed already in the external coincidence model.

One such point is, how the oscillator is exactly synchronized by the light-dark cycle. In the example of a short day plant as shown in figure 20.25. Bünning assumed the switch from darkness to light to phase set the clock (the photophilic phase, red in the figure, coincides with lights on in the long day *and* in the short day condition). However, this does not need to be so. Nei-

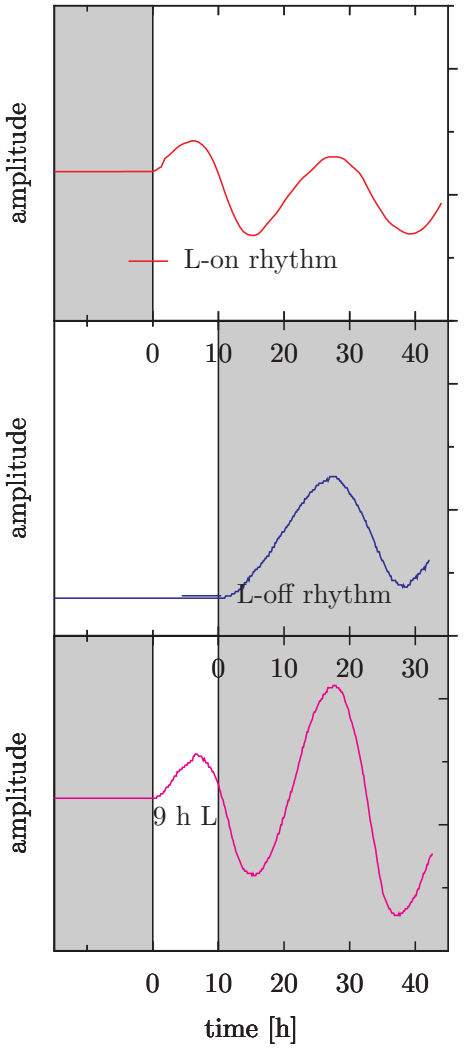

Figure 20.28: *Internal coincidence model of photoperiodic induction of flowering. Light-on rhythm (red) of* Kalanchoe *flowers after two weeks of DD (gray) released into continuous light LL (unshaded). Maxima of light-on rhythm at 6 and 27 hours after onset of light. Light-off rhythm (blue) after release from a few days LL (no shading) into DD (shaded). Maximum 18 hours after onset of DD. Magenta: Flowers in DD, then 9 hour light and back into DD. Both rhythms superimpose, amplitude is increased. Longer and shorter light periods would lead to smaller amplitudes (not shown).* Kalanchoe *is maximally induced to flower by 9:15 LD (depicted here).*

ther does the lights off part need to set phase. It is more likely, that this entrainment is more complicated. We will come back to it (see figure 20.29).

A second point of vagueness is which part of the internal oscillator cycling has to coincide in which way with the light-dark cycle. This too has been addressed in the external coincidence model, but insufficiently.

We ([126]) have used a feedback model developed first for the description of ultradian rhythms and later for circadian rhythms ([717], [745]) to clarify these uncertainties. The model oscillator is driven by light-dark cycles (done on an analogue computer, where different light-dark schedules were fed into the program) and the resulting curves were recorded (see figure 20.29). A series of experiments using *Chenopodium rubrum* flower induction were performed parallel to the simulations in which the same combinations of light-dark cycles were used. We tried to find from the simulations an indicator of photoperiodic induction. The distance between *light on* of the LD cycles and the closest minimum of the oscillation (the sign was disregarded) was called ψ and the mean values of all ψ´s turned out to be a fairly good predictor of photoperiodic induction (see figure 20.30 and figure 20.31).

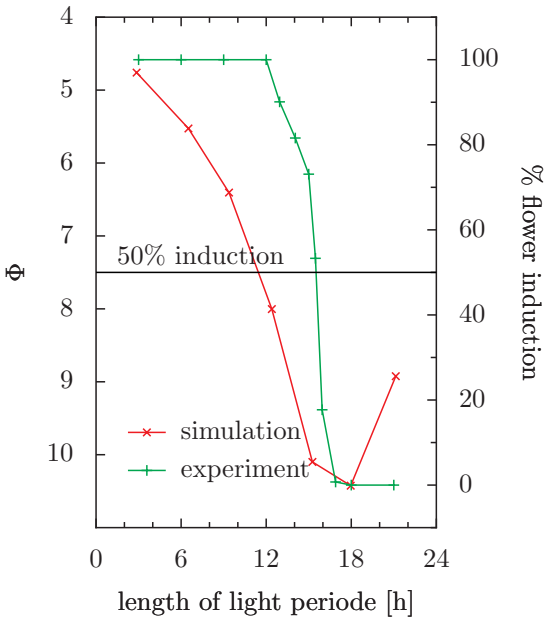

Figure 20.30: *Simulation of oscillations and photoperiodic experiments under cycles of various light periods (x-axis) and corresponding dark periods in 24 hour cycles.* Chenopodium rubrum *plants ecotype 374 were grown in continuous light and transferred to three cycles of various LD schedules. The percentage of flowering plants is shown (green curve) alongside with the ψ values from the simulations (red curve). High flower induction occurs up to 12 hours, no induction beyond 16 hours. The ψ curve shows similarities in its time course. See details in [126]*

20.17 Fragrance rhythms

Flowers are organs of plants which in many cases serve to attract pollinators such as insects or birds or bats. Fragrances are emitted from flowers often during certain times of the day or night only. Flowers which are pollinated by night active moths open therefore the flowers at night and de-

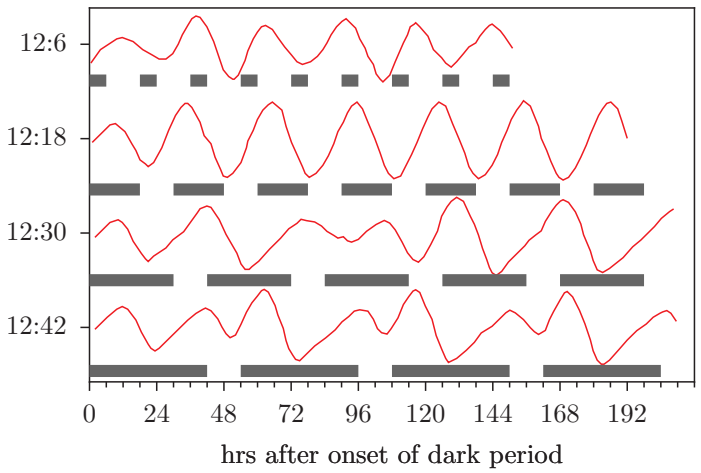

Figure 20.29: *Simulation of oscillations with a feedback model (see figure 8.12, where 'disturbance' would be light) using different light-dark cycles as shown on the y-axis (LD 12:6, 12:18, 12:30, 12:42 hours). The model oscillator is driven by light-dark cycles (done on an analogue computer, where different light-dark schedules were fed into the program) and the resulting curves were recorded. The distances ψ between light on (change between gray and white area) to the closest minimum were shown to be an indicator of photoperiodic induction (see figures 20.30 and 20.31). After [126]*

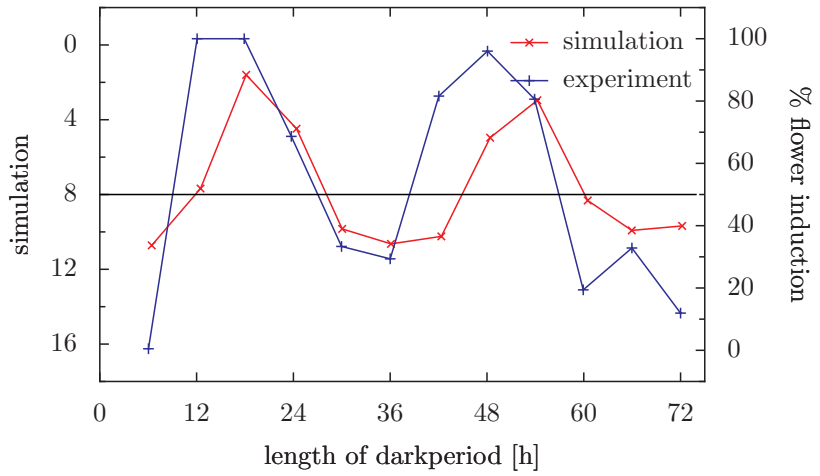

Figure 20.31: *Simulations (red curve) and experiments (blue curve) on flower induction in* Chenopodium rubrum *plants ecotyope 374 under LD-schedules consisting of a 6 hour light period and various dark periods (abscissa). The degree of flower induction (right y-axis) and the ψ values (left y-axis) show a similar pattern. After [126]*

liver fragrance.

In some studies it was shown that this emission of scent is controlled by an endogenous rhythm. It is mainly found in plants which flower during the night. In plants which flower during the day the rhythm is driven by the light-dark cycle and not by an endogenous rhythm. To the night flowering plants belong a number of *Caryophyllaceae*. The ecology of flowering and the pollination mechanisms in *Caryophyllaceae* are presently studied intensively by [727]. The fragrance of *Saponaria officinalis* has been established by [1069] in a diploma thesis using headspace-methods and gas chromatography. The plant is found at ruderal places especially in meadowland in whole Europe (not in the alps). It flowers from late summer to the late fall. It is very fragrant in the evening and the night. Anthesis begins between 20:00 and 22:00 clock. The flowers stay open during the day also (in contrast to *Silene nocturnum*, where the flowers close during the day).

To collect samples of the fragrance, a special apparatus was used ([967]), which is shown and explained in figure 20.32. Parallel to measuring the fragrance the flowers were recorded with a video camera. The fragrance substances are eluted from the filter tubes with carbon disulfide and analyzed by a gas chromatograph. From the chromatograms the kind and amount of fragrant substances can be determined for the different time sections. Such a gas chromatogram of the headspace of *Saponaria officinalis* is plotted in figure 20.33. The fragrance consists of 75% methyl benzoate, about 5% benzaldehyde and 2% benzylcyanide. About 40 further substances are detectable in small amount, which were not identified. *Saponaria officinalis* emits methyl benzoate and benzalde-

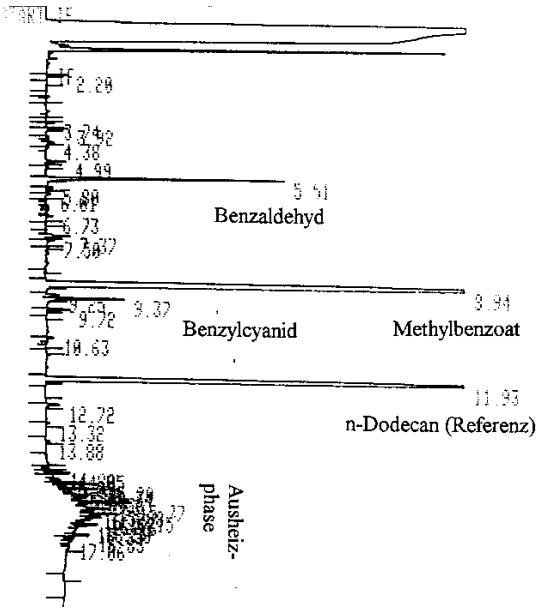

Figure 20.33: *Gas chromatogram of the headspace of* Saponaria officinalis *with three main components: Benzaldehyde, methyl benzoate and benzylcyanide. As a reference n-dodecan was used. The phase of 'heating out' is marked*

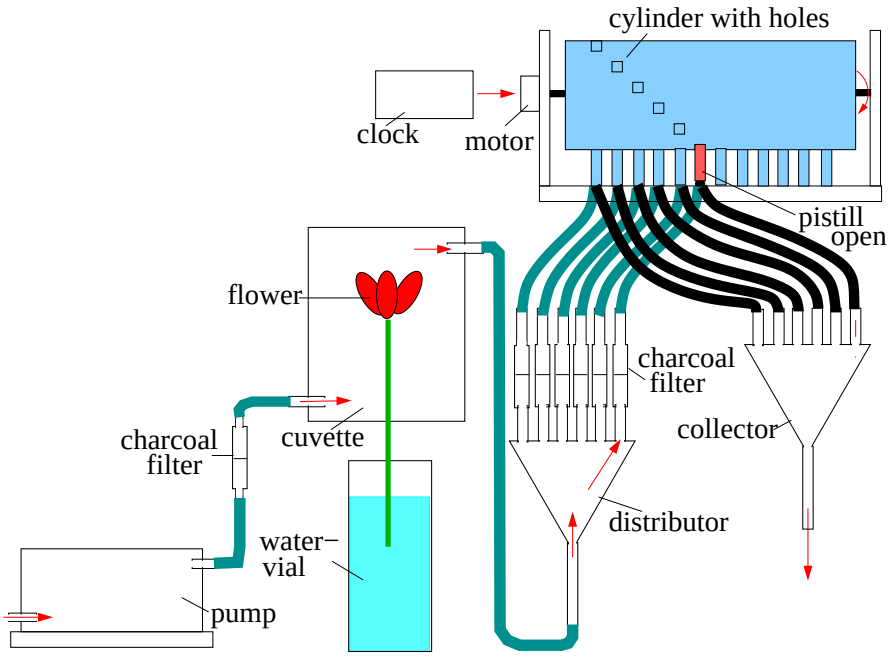

Figure 20.32: *Apparatus for collecting fragrance samples at different times of the day. The flowers are confined to a cuvette. Air is pumped through which is cleaned by an active charcoal-filter before entering the cuvette. The fragrance substances emitted by the flowers are taken up by the air and absorbed on the active charcoal in filter tubes. Twelve filter tubes are applied in a star-like arrangement on top of the cuvette. A roller opens for a certain time (timer, relay) one of the silicon tubes. In this way the fragrance of a certain time section (for instance 3 hours) is collected in one of the filter tubes. Afterward the next filter tube is opened and collects the fragrance for the next time section. After all filters have been loaded, a new set of filter tubes are mounted and the recording continued. Behind the roller the tubes are united to one tube again after passing a star-like collector. The air stream is passing also a rotameter which measure and keeps constant the speed of the air stream*

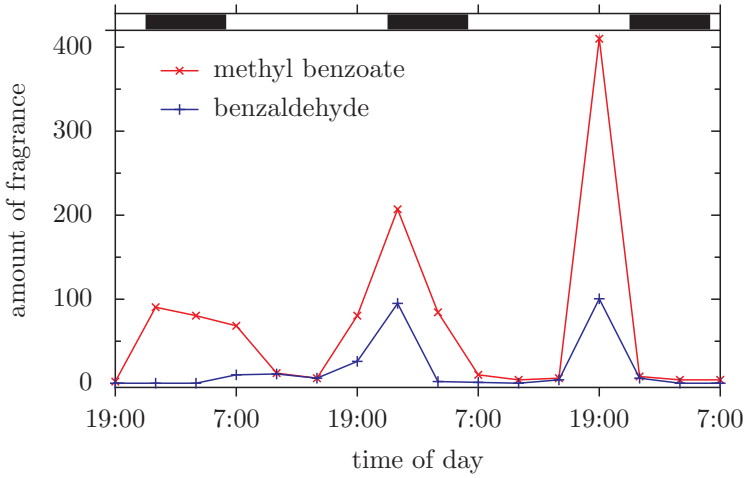

Figure 20.34: *Rhythmic emittance of methyl benzoate and benzaldehyde by flowers of* Saponaria officinalis *in the light-dark-cycle (first maximum, dark period marked by a dark line, light period by a bright line above the curve) and in continuous light (right part of the figure). After [1069]*

hyde during the light-dark-cycle as well as under continuous light rhythmically (figure 20.34). Benzylcyanide was not at all times identifiable. The main component methyl benzoate has its maximum during the dark period around 24:00 clock. In continuous light the second maximum occurs later (at 3:00 clock). The free run period would thus amount to about 27 hours. In the master thesis details are described and some observations which would be worth to follow up. For instance, the rhythm of the benzaldehyde might have a slightly different free run period as that of methyl benzoate. The two substances might have different functions in attracting the pollinators.

20.18 Arrhythmia

Oscillations can be disturbed by external influences such as a kick in the case of a swinging swing. Its effect depends on the phase at which this kick occurs. In biological rhythms such as circadian clocks those external influences are for instance light or temperature pulses and several examples were given. The effect of these pulses on the rhythm as a function of the time of application (phase) is usually shown in phase response curves (for examples see figures 4.9, 6.15, 8.9, 8.6, 14.6, and 16.4).

It was found first in the case of the eclosion rhythm of *Drosophila pseudoobscura* and later in other organisms such as the petal movement rhythm of *Kalanchoe* that with a strong disturbing pulse there is a certain phase point at night (subjective midnight) where pulses delay the rhythm if given before and advance the rhythm if given after this point. However, if given exactly at this point and in a certain strength the rhythm might disappear. This critical pulse is at the lowest range of the strong pulses and at the highest range of the weak pulses. The difference between a strong and a weak pulse

is explained in figure 14.6 for the eclosion rhythm in *Drosophila* ([1575]).

20.19 Genetics of circadian rhythms

Already in 1932 Bünning has shown that the leaf movement rhythm o varieties of bean populations differ in their period length by 3 hours. This difference stayed constant when each of the varieties was crossed with itself for four generations. Crossing between the varieties did not change the distribution of the period lengths. This indicated a polygenic inheritance ([179]).

[170] studied the circadian rhythm of phototactic reactions in *Chlamydomonas*. These alga are favorable for genetic studies because they are haploid, possess a small genome of about 100 Mb and because numerous mutants are available (recent review see [569]). Already the wild type shows variations in the period length. Using mutagenesis, in 0.5 to 2% the period length was changed. In most cases the period was longer as compared to the wild type. [171] isolated four mutants, per-1, per-2, per-3 and per-4. The period lengths were between 26 and 28 hours. The mutations were on four different loci and crossings showed that per-1 was dominant, per-2 was recessive and per-4 was semi-dominant ([173]). Semi-dominant are also all mutants of *Drosophila* and *Neurospora crassa*. The period lengths of the crossings were up to 40 hours. The effects are according to [172] additive [18] (if the mutant A has a period length of $\tau_{WT} + n$ and B a period length of $\tau_{WT} + m$, the double mutant AB has a period length of $\tau_{WT} + (n + m)$). If true, the four mutants should influence the same circadian system. However, according to [847] it is more likely that the effects are multiplicative ($\tau_A * \tau_A / \tau_{WT}$). There are no epistatic interactions.

Metabolic mutants of *Neurospora crassa* were checked for their period lengths. In 10-15% of the cases deviations from the period of the wildtype were found ([264], [850]). Interesting were especially mutants in the fatty acid metabolism. They in turn are important for membranes and their properties. The lipid-mutants fat1, alt1 and fad4-7 belong to these cases.

A more recent review of the genetic studies and results of circadian rhythms is by [1399]. *Arabidopsis thaliana* was and is genetically studied intensively (review [1004]). After the introduction of a circadian hand (cab2::luc) into the genome of this plant the circadian rhythm of amplitude of cab2 transcription rate of the amount of RNA could be recorded non-invasively as luminescence with a sensitive video camera. The period length under continuous light is 24.5 and under darkness 30 to 36 hours.

These differences in period length could be used to isolate blind mutants, because they showed in spite of continuous light a longer period. The mutant hy1 which does not possess a chromophor for phytochrome, exhibits under continuous light the period length of the wild type of 24.5 hours. This indicates light inputs into the circadian clock via multiple photoreceptors (see page 20.13.1). In continuous blue light the period length is shorter as in the wild type. Phytochrome and photoreceptors must therefore interact with each other in order to produce the period of the wild type. The det1-1 mutants are under

[18] as was found also in *Neurospora crassa* ([337], [425])

darkness as if in continuous light. This is not only true for photomorphogenesis, but also for the circadian clock. In this mutant something is missing which would make the clock run faster. It was shown that this factor is in the light input pathway and that therefore DET1 is not a central component of the clock. The mutant cop1-6 reacts similarly. The mutant det2 however shows the circadian behavior of the wild type. In this mutant an enzyme of the blue receptor pathway is affected ([892]); the blue receptor pathway is thus not an essential part of the clock.

Finally mutants were used, in which the flowering time differs from the wild type. Since the photoperiodic time measurement uses a circadian clock ([180]), those mutants are candidates for differences in the circadian system. Mutants of this type are elf (earlier flowering), day neutral mutants (esd4, period length shorter as in the wild type), toc1 and the late flowering mutant CO. In elf, however, the input to the clock is affected, in CO the output from the clock.

20.20 Time series analysis

The analysis of rhythms uses methods called time series analysis. Unfortunately the literature is often quite special and not easy to follow for the layman. Visual inspection of the data is the first step ([1207]), but often the rhythm is hidden in the data and special methods have to be applied. For instance, [514] studied stomata-rhythms in *Vicia faba* epidermis pieces and showed how to extract information on an circadian rhythm out of messy data by using different procedures such as moving average for smoothing the raw data and trend removal. Maximum entropy spectral analysis (MESA, [1496]) was used here as well as in other more recent papers as an alternative method to the standard Fourier- and periodogram analysis methods which are usually applied. Often used is the chi-square periodogram of [1395]. For new approaches to analyse time series see [1275]. Some publications are recommended which help to get familiar with the different methods used in time series analysis: [1031], [393], [398].

Practical examples for time series analysis of biological rhythms are given by [331], [8], [325].

Recording of locomotor activity in animals is a frequently used method to obtain data on the rhythmic control (see for instance figure 3.1). Here special methods are used ([333]). [789] has compared six different methods to measure period of circadian rhythms.

Most time series analysis programs work on rhythms only which are stationary: Their period length should not vary with time. However, this is often not the case in biological rhythms. Here the complex demodulation method is recommended ([1390]). It shows the varying periods and possible phase shifts in a plot which resembles an actogram in which the daily onsets of activity are marked and connected by a straight line (if the period stays constant), by two or more straight lines (if phase shifts occur) or by a curve representing the changing period.

Special methods are used to analyse the orientation of animals by using sun or moon compass abilities ([66], [1331])

Time series analysis program packages for computers are available ([961], [960], [962], [324], [970]). The book by [1176] contains short descriptions and programs for time series analysis.

For literature on time series analysis see

[398], [1143], [821].

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