

**The role of an environmental gradient in driving
population divergence in common gobies
(*Pomatoschistus microps*)**

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ISABEL MAGDALENA MÜCK

aus Bad Reichenhall

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Dekan:

Prof. Dr. Wolfgang Rosenstiel

1. Berichterstatter:

Dr. Katja Heubel

2. Berichterstatter:

Prof. Dr. Katharina Foerster

“There is no substitute for careful and intensive field work if one wants to find out what is happening in natural populations.”

Endler, 1986

Table of Contents

Zusammenfassung.....	7
Summary	8
Introduction.....	9
Environmental-dependent sexual selection.....	9
Neutral versus adaptive evolution	10
Environmental gradients	11
The Baltic Sea	12
The common goby (<i>Pomatoschistus microps</i>)	14
Study goals	16
(I) Ecological variation along the salinity gradient in the Baltic Sea and its consequences for mating success in common gobies	16
(II) Population divergence in common gobies: neutral or adaptive evolution?.....	17
(III) Gonadal investment in common gobies along the salinity gradient in the Baltic Sea.....	18
CHAPTER I	19
Ecological Variation along the Salinity Gradient in the Baltic Sea and its Consequences for Mating Success in Common Gobies.....	20
Abstract	20
Introduction.....	21
Methods	24
Study species	24
Field study design	25
Habitat survey	25
Nesting resources	26
Population density and sex ratios	28
Body size.....	29
Mating assay.....	29
Data analyses.....	30
Results	32
Nesting resources	32
Population density.....	35
Operational sex ratio	35

Body size.....	36
Male body size and size of <i>Mya arenaria</i>	37
Mating assay.....	38
Brood characteristics.....	40
Discussion.....	42
Conclusion.....	48
Acknowledgements.....	49
CHAPTER II.....	50
Population Divergence in Common Gobies: Neutral or Adaptive Evolution?.....	51
Abstract.....	51
Introduction.....	52
Methods.....	56
Study species.....	56
Fish collection and sampling sites.....	56
Genetic analyses.....	59
P _{ST} - F _{ST} comparison.....	62
Results.....	64
Genetic analyses.....	64
P _{ST} - F _{ST} comparison.....	72
Discussion.....	73
Genetic structure of common gobies on coarse and fine spatial scales.....	73
Neutral versus adaptive evolutionary processes driving population divergence.....	75
Roles of natural selection versus genetic drift on phenotypic population divergence in Baltic common gobies: a P _{ST} – F _{ST} comparison.....	77
Conclusion.....	79
Acknowledgements.....	80
Supplemental data.....	81
.....	83
CHAPTER III.....	83
Gonadal Investment in Common Gobies along the Salinity Gradient in the Baltic Sea.....	84
Abstract.....	84
Introduction.....	85
Methods.....	88
Study species.....	88
Sample collection.....	89
Fish dissection.....	91

Data analysis.....	92
Results	96
Males	96
Differences in gonadal and liver investment between salinity levels	96
Differences in gonadal and liver investment between male categories.....	101
Females.....	103
Differences in gonadal and liver investment between salinity levels	103
Differences in gonadal and liver investment between female ripeness stages.....	105
Gonad and liver mass	107
Discussion.....	108
Female mimics in common gobies	108
The relationship of gonad size and salinity	110
The relationship of liver size and salinity	111
Conclusion	112
Acknowledgements.....	113
Summary and discussion	114
Synopsis.....	119
Outlook.....	121
Chapter I:.....	121
Chapter II:.....	122
Chapter III:.....	122
List of contributors and affiliations	123
Acknowledgements / Danksagung	124
References.....	126

Zusammenfassung

Umweltgradienten stellen eine außerordentliche Möglichkeit dar, um Adaption und Evolution im kleinen Maßstab zu studieren. Entlang solcher Gradienten fluktuieren abiotische und biotische Faktoren in Raum und Zeit, und erlauben es daher die Verbreitung von Organismen zu erforschen, in Bezug auf deren Toleranz und Adaption gegenüber der Umwelt. Im Vorfeld meiner Arbeit war nur wenig darüber bekannt wie sich innerhalb einer Art Unterschiede in der Ökologie entlang von Umweltgradienten auf die Variation von Paarungserfolg, Populations-Divergenz und physiologische Parameter, welche den Reproduktionserfolg bestimmen, auswirken. Diese Themen habe ich anhand von Strandgrundeln (*Pomatoschistus microps*) entlang des Salzgehaltsgradienten in der Ostsee untersucht. Zuerst habe ich die tatsächlichen ökologischen Unterschiede denen Strandgrundel Populationen in der Ostsee ausgesetzt sind erfasst. Genau genommen habe ich Habitats Untersuchungen durchgeführt und Daten zur Verfügbarkeit von Nestmaterial (Muschelschalen), Demographie und phänotypischen Unterschieden zwischen Strandgrundel Populationen entlang des Salzgehaltsgradienten in der Ostsee aufgenommen (Kapitel I). Ich habe herausgefunden, dass Salzgehalt deutlich mit Nestmaterial Qualität und Quantität, der Populationsdichte sowie der Körpergröße von *P. microps* korreliert. Daraufhin habe ich untersucht, ob es Anzeichen lokaler Adaption der einzelnen Populationen gibt (Kapitel II). Es gelang mir die Existenz einer klaren genetischen Struktur in Strandgrundeln zu zeigen, sowohl im großen als auch im kleinen geographischen Maßstab. Außerdem konnte ich zeigen, dass divergierende Selektion phänotypische Populationsunterschiede in Baltischen Strandgrundeln antreibt (Kapitel II). Schlussendlich habe ich das Gonaden- und Leberinvestment in Strandgrundeln entlang des Salzgehaltsgradienten in der Ostsee untersucht (Kapitel III). Das Investment in beide diese Organe ist mit der Reproduktionsleistung verbunden. Männchen die aus Habitaten mit niedrigem Salzgehalt stammten hatten wesentlich größere Gonaden (sprich akzessorische Geschlechtsdrüsen) und Lebern als Männchen aus Habitaten mit mittlerem oder hohem Salzgehalt. Mit meiner Arbeit konnte ich zeigen, dass Salzgehaltsunterschiede in der Ostsee zur plastischen Variation (Chapter I, III) in Strandgrundel Populationen führen, mit Anzeichen von genetisch manifestierter Differenzierung (Chapter II).

Summary

Environmental gradients constitute an extraordinary opportunity to study adaptation and evolution on a small spatial scale. Along such gradients abiotic and biotic factors fluctuate in space and time and allow studying the distribution of organisms in terms of environmental tolerances and adaptations. Prior to my research there was little knowledge on how differences in the ecology across an environmental gradient influence variation in mating success, population divergence, and physiological parameters determining reproductive success within a single species. I studied these topics in common gobies (*Pomatoschistus microps*) along a salinity gradient in the Baltic Sea. I first described the actual ecological differences between populations of common gobies. Specifically, I conducted habitat surveys collecting information on nesting resources (empty mussel shells) availability and assessed demographic features as well as phenotypic differences between common goby populations along the salinity gradient in the Baltic Sea (Chapter I). I found that salinity correlated with nest resource quantity and quality, population density and body size of *P. microps*. I further investigated whether there is any evidence that populations are locally adapted (Chapter II). I demonstrated a clear population genetic structure on a coarse and fine geographic scale, likely driven by divergent selection. Subsequently, I assessed gonad and liver investment of common gobies along the salinity gradient. The investment in both of these organs is linked to the reproductive output. Males originating from low salinity sites had significantly larger gonads (specifically sperm duct glands) and livers than males from intermediate or high salinity sites (Chapter III). With my research I could show that differences in salinity in the Baltic Sea lead to plastic variation (Chapter I, III) in common goby populations, with signs of genetic population divergence (Chapter II).

Introduction

The power of selection to produce adaptations of organisms to their environment, resulting in the magnificent phenotypic diversity observed in nature, continues to astonish scientists and non-scientists alike. Selection is usually divided into natural and sexual selection (Darwin 1871; Andersson 1994). However, a clear categorisation is often difficult and cause for an ongoing debate in this field (Lyon and Montgomerie 2012). Both, natural and sexual selection take place in spatial heterogeneous environments. While fluctuations of environmental conditions in natural selection have long been considered (Endler 1986), studies examining environmental influences on the agents of sexual selection are still less common (Cornwallis and Uller 2010; Janicke et al. 2015). However, more and more evidence is found that sexual selection may be stronger than natural selection leading to divergent sexual selection driving adaptive population divergence (Svensson et al. 2006; Labonne and Hendry 2010).

Environmental-dependent sexual selection

Sexual selection is often assumed to be consistent. However, mounting evidence suggests that the strength and direction of sexual selection can fluctuate over space, time, or context (Cornwallis and Uller 2010; Siepielski et al. 2011). Much of the existing work on plasticity in sexual selection focuses on differences in social environments, such as population density and sex roles (CluttonBrock et al. 1997; Levitan 2004; Aronsen et al. 2013; Wacker et al. 2013). However, some examples demonstrate that the sexual selection regime can vary in space and time irrespective of demographic factors (Moblely and Jones 2009; Byers and Dunn 2012). This highlights the importance of including ecological factors in order to fully understand how sexual selection operates (see for a review Miller and Svensson 2014). One example is environmental context-dependent mate choice of females (i.e., plasticity in female

preferences). Females alter their mating decisions depending on for instance resource quality (Gillespie et al. 2014) or predation pressure (Godin and Briggs 1996). In addition to plasticity in mate preferences, sexually selected traits themselves are also expected to be phenotypically plastic, serving as indicators of an individual's condition. Most approaches examining sexual traits, however, overlook that the evolution of sexually selected traits is determined by the interplay between environmental heterogeneity and phenotypic plasticity, which changes over time and space causing fluctuating selection on such traits (Cornwallis and Uller 2010; Miller and Svensson 2014).

Generally, studies on sexual selection often do not consider ecological factors. This is most likely because of the difficulty to assess the complex interactions between individuals and environments. Thus, a clear prediction about how sexually selected phenotypes evolve is often difficult. However, there can be no doubt that environmental factors are major determinants of an individual's reproductive performance, affecting the process and outcome of sexual selection (Robinson et al. 2012).

Neutral versus adaptive evolution

It is known that micro-evolutionary responses to spatial variation in the environment can be caused by adaptive- (i.e., natural- and/or sexual selection) and neutral-processes (e.g. mutations, genetic drift), however, their relative roles often remain unknown (but see e.g. Brousseau et al. 2015). Adaptive and neutral processes may interact in several different ways. For instance natural selection may lead to adaptive population divergence (i.e., genetically based phenotypic differences that improve local fitness) in different environments. The dispersal (i.e., gene flow) of individuals between those environments may, however, homogenize the gene pool and thus oppose adaptive divergence (Hendry et al. 2001;

Lenormand 2002; see for a review Räsänen and Hendry 2008; Servedio and Kopp 2012). Neutral genetic markers provide a cost effective and often used way to study population divergence (Gaggiotti et al. 2009). Divergence in these markers is caused by genetic drift as a result of reduced gene flow between populations. Gene flow can be influenced by the landscapes by two major processes: isolation by dispersal limitation (IBDL) resulting in a pattern of (1) *isolation by distance* (IBD; Wright 1931) and/or isolation by adaptation (IBA) resulting in a pattern of genetic differentiation known as (2) *isolation by environment* (IBE; Nosil 2009; Orsini et al. 2013). IBD is an evolutionary neutral process without selection acting on advantageous traits of individuals and genetic drift is caused by reduced gene flow due to great geographic distances or physical barriers (= isolation by resistance; Orsini et al. 2013) between populations. IBE on the other hand describes an adaptive evolutionary process, whereby divergent selection favours certain traits in individuals of ecologically different environments. Thus, reduced establishment success of immigrants may occur due to maladaptation. This can lead to reduced effective gene flow among populations inhabiting ecologically dissimilar habitats. To assess processes structuring population genetic variation in natural landscapes the interpretation of patterns of genetic differentiation along spatial and environmental gradients is highly suitable.

Environmental gradients

Environmental gradients are extremely valuable to study evolutionary-ecological interactions. Fluctuations of abiotic and biotic factors and the often continuous distribution (i.e., no barriers) of species along such gradients, facilitate for instance the investigation of modes of population divergence. Genetic and phenotypic variation as a function of environmental variation and adaptation can be investigated at geographically large scales along environmental gradients (Doyle et al. 2010; Jennings et al. 2013). The goal for understanding

biological variation along environmental gradients, is determining whether population divergences represent the outcome of phenotypic plasticity or local genetic adaptation due to divergent selection (Lande 2009; Storz et al. 2010; see Chapter II) and the relative role of gene flow in these processes (Hendry et al. 2002; Hendry and Taylor 2004; Crispo et al. 2006; Crispo 2008). Fluctuations of abiotic and biotic factors along environmental gradients can determine the population genetic structure (Nanninga et al. 2014), facilitate speciation (Doebeli and Dieckmann 2003), cause differences in intraspecific population dynamics (Peterman and Semlitsch 2013), and influence species composition and diversity (Zettler et al. 2014). The geological and recent history is packed with examples of species that failed to adapt and went extinct during periods of rapid environmental change (Bell and Collins 2008; Chevin et al. 2010; Fakheran et al. 2010). Thus, it is surprising how little attention has been paid by evolutionary ecologists to the understanding and awareness of constraints and trade-offs that suppress the evolution of rapid adaptation.

Environmental gradients occur in terrestrial landscapes, where latitudinal gradients together with temperature gradients are among the most well studied examples showing the immense impact that changing abiotic parameters can have on organisms (e.g. Naya et al. 2011). Gradients also occur in aquatic environments, such as: depth- (Ota et al. 2012), temperature- (Doyle et al. 2010), salinity- (Hampel et al. 2005) and food-gradients (Emlen 2008). A natural aquatic arena offering steep environmental gradients is the Baltic Sea. Thus, the Baltic Sea is highly suitable to study population divergence in marine organisms.

The Baltic Sea

The Baltic Sea (Fig. 1) is one of the world's largest brackish inland seas and came into existence only ~ 10,000 years before present. It was completely covered by ice during the late

Pleistocene. After the ice sheet began to retreat, four stages are recognized during the postglacial progression of the Baltic basin: (1) the Baltic Ice Lake (freshwater), which drained to sea level creating the (2) Yolida Sea (brackish) followed by the (3) Ancylus lake, a freshwater lake isolated from the sea due to land uplift, and the (4) Littorina Sea, a second brackish stage initiated due to a dramatic sea level rise (Björck 1995). The Baltic Sea in its current stage is only ~ 2,000 years old. Not only its complex salinity history attracts ongoing scientific interest, but also its characteristic salinity gradient as it exists today; salinity ranges from 1 to 2 PSU (Practical Salinity Units) in the innermost parts to up to 25 PSU at the entrance (i.e., the Skagerrak) to the North Sea (HELCOM 1996; Fig. 1). In this species-poor and trophically simple ecosystem, many species currently find their distribution limits across these salinity gradients (Bonsdorff and Pearson 1999; Westerborn et al. 2002; Jansen et al. 2009). Populations of several Baltic species are genetically differentiated, often with clearly distinct northern marginal populations, where low salinity acts as a barrier for gene flow (Johannesson and Andre 2006; Holmborn et al. 2011; Olsson et al. 2012). It is thus not surprising that populations of some species occurring in the Baltic Sea are genetically different from populations inhabiting the North Sea or Atlantic (e.g. Nilsson et al. 2001; Sjöqvist et al. 2015). The Baltic Sea with its young age and salinity gradient offers a suitable natural arena to investigate rapid evolution by examining recent population genetic events and adaptive phenotypic divergence between populations on a fine geographic scale.

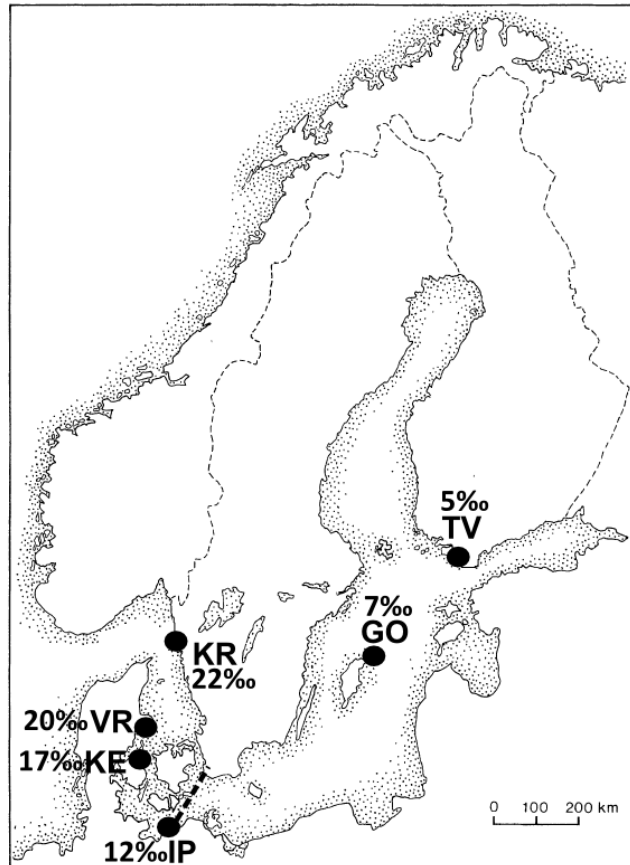


Figure 1. The Baltic Sea. Shown are the six main sampling sites from Chapter I and III including average salinity (PSU) measured during data collection in 2012, 2013 and 2014. The dashed line from Falsterbo (south Sweden) to Travemünde (Germany) is marking the entrance of the Baltic Sea proper. See Table 1 for site abbreviations. Map modified after Forsgren et al. 1996.

The common goby (*Pomatoschistus microps*)

The common goby (*Pomatoschistus microps*) is a marine fish, which is found in shallow soft-bottomed marine and estuarine habitats from southern Norway to Portugal, including the Baltic Sea (Wheeler and Du Heaume 1969). It is sexually dimorphic (see Fig. 2) and between 3-4 cm (total length, TL) in the Baltic Sea, ~ 6 cm on British coasts and ~ 5 cm in the Mediterranean (Jones and Miller 1966; Bouchereau and Guelorget 1998). Due to its broad distribution this goby species experiences a higher degree of environmental heterogeneity than most of the other European Gobiidae (Wallis and Beardmore 1984). The common goby

Introduction

has a short lifespan with a maximum of 21-26 months, but most adult fish die after their first breeding season with 12-20 month (Miller 1975). In the Baltic Sea common gobies migrate in spring (May-June) from deeper waters into very shallow, sheltered breeding habitats and starts reproducing (Vestergaard 1976). When water temperatures in autumn fall below 5°C, Baltic common gobies migrate back into deeper waters (Jones and Miller 1966; Vestergaard 1976). Compared to the closely related sand- (*P. minutus*) or marbled-goby (*P. marmoratus*), common gobies seem to be better adapted to shallow inshore areas where water temperatures are high during summer months, as well as to highly variable salinity conditions (Fonds and van Buurt 1974; Rigal et al. 2008).



Figure 2. Sexual dimorphic female (on top) and male common goby (*Pomatoschistus microps*) caught in a high salinity population within the Baltic Sea in 2013 (size standard = 0.5 cm).

Study goals

The central aim of my study was to evaluate how variation of an abiotic factor (i.e. salinity) along an environmental gradient influences fundamental aspects (i.e. nest availability, population density, body size, gonadal investment) of mating success, possibly driving population divergence in common gobies (*Pomatoschistus microps*). To do so, I conducted a geographically extensive field study over three consecutive years along the salinity gradient (West to East) in the Baltic Sea collecting data on habitat structures, demography, morphology and genetics of common gobies. Specifically, I studied the following topics:

(I) Ecological variation along the salinity gradient in the Baltic Sea and its consequences for mating success in common gobies

Little is known on how ecological consequences evoked by salinity are affecting fundamental aspects of the life history such as the demography, morphology as well as mating- and reproductive success within a species. I first assessed nesting resource (empty mussel shells) quantity and quality along the Baltic salinity gradient from West (high salinity) to East (low salinity). A nest site is the most essential prerequisite for mating success in a mussel-breeding fish like the common goby and therefore, mussel shell availability is a limiting factor for mating success. I also examined demographic factors like population density and sex ratios, which are known to affect the strength and direction of sexual selection. To directly assess how spatial variation of salinity affects the morphology of *P. microps* I compared body size between populations. Finally, to link ecological, demographic as well as morphological differences

between populations to modes of sexual selection I conducted a field based mating assay with size-standardized nests along the salinity gradient to compare variation in mating- and reproductive success.

I expected low salinity sites to constitute a suboptimal habitat for mussel shells leading to reduced nest availability and thus mating success, which should be correlated to lower population density of common gobies. Furthermore, I predicted common gobies along the salinity gradient to show differences in body size, with individuals of low salinity to be smaller due to energy allocation caused by high costs for osmoregulation.

(II) Population divergence in common gobies: neutral or adaptive evolution?

While assessing spatial ecological differences between populations is a prerequisite to better understand the process and outcomes of sexual selection (Chapter I), population genetic analyses are required to assess the role and contribution of natural selection driving population divergence. I aimed to investigate the genetic structure of Baltic common gobies and the relative roles of neutral (i.e., isolation by distance, IBD) and adaptive (i.e., isolation by environment, IBE) evolution driving population divergence. I did so by 1) contrasting IBD with IBE using neutral genetic markers and 2) by comparing phenotypic differentiation (P_{ST}) with neutral genetic differentiation (F_{ST}).

I expected to find a genetic differentiation between western and eastern Baltic common goby populations due to adaptation to salinity resulting in a pattern of IBE. Furthermore, I expected to find phenotypic differentiation between western and eastern Baltic populations driven by

divergent selection favouring different phenotypes in different (salinity) environments ($P_{ST} > F_{ST}$).

(III) Gonadal investment in common gobies along the salinity gradient in the Baltic Sea

After investigating population divergence in Baltic common gobies (Chapter II) I moved on to assess how salinity affects gonadal and liver investment, both essential processes determining reproductive outcomes. Variation in gonad and liver mass along the salinity gradient may suggest divergent selection favouring different phenotypes in different environments. I assessed testes mass and sperm duct gland (SDG) mass separately to assess differential allocation of investment into gonads depending on salinity. I also included females to compare possible patterns of gonadal and liver investment variation between sexes evoked by salinity.

I expected to find differential allocation of investment into gonads and liver between high and low salinity sites. Specifically, with males of low salinity sites investing more in protective mucus producing SDGs than in testes due to low salinity bearing a higher risk of egg infection. Furthermore, individuals of low salinity sites investing more in livers (i.e., energy stores) due to high costs of osmoregulation.

CHAPTER I

Ecological Variation along the Salinity Gradient in the Baltic Sea and its Consequences for Mating Success in Common Gobies

I. M. Mück, K. U. Heubel

Abstract

Although it has become clear that sexual selection may shape mating systems and drive speciation, the potential constraints of environmental factors on processes and outcomes of sexual selection are largely unexplored. Here, we investigate the geographic variation of such environmental factors, more precisely the quality and quantity of nest resources along a salinity gradient in the Baltic Sea, and test whether we find any correlated morphological differences in body size between populations of common gobies (*Pomatoschistus microps*), a small marine fish with a resource based mating system. In a geographically extensive field study we sampled five populations of *P. microps* occurring along the salinity gradient (decreasing from West to East) in the Baltic Sea over three consecutive years. Nest resource quantity and quality decreased from West to East, and a correlation between mussel size and male body size was detected. Population density, sex ratios, reproductive success and brood characteristics also differed between populations but with a less clear relation to salinity. With this field study we shed light on geographic variation of distinct environmental parameters possibly acting on population differentiation. We provide insights on relevant ecological variation, and draw attention on its importance in the framework of environmental-dependent sexual selection.

Introduction

Many aspects of an organism's social organisation can be predicted if the limits of its environment are known. Environmental factors can, for example, determine to which degree mates and/or resources can be defended and monopolised, and such ecological constraints impose limits on the degree to which sexual selection can operate (Emlen and Oring 1977; Forsgren et al. 1996b; Gillespie et al. 2014). It is well known that sexual selection is influenced by environmental factors (Hill 1994; Møller 1995; Kwiatkowski and Sullivan 2002; Gamble et al. 2003; Cornwallis and Uller 2010; Gillespie et al. 2014) but surprisingly little attention has been paid to environmental-dependent sexual selection causing variation of sexual selection over space, time, or context (but see Almada et al. 1995; Forsgren et al. 1996b; Siepielski et al. 2009; Janicke et al. 2015).

An individual's access to mates and resources within a population depends strongly on its competitive ability (Parker and Sutherland 1986). Large male body size is usually selected for in male contest and/or by female choice, and generally increases a male's competitive ability (reviewed in Andersson 1994). Both competitive ability as well as resource availability are shaping sex role dynamics and mating systems and are therefore strongly related to processes and outcomes of sexual selection (Emlen and Oring 1977; Kvarnemo and Ahnesjö 1996); the level of competition is determined by resource availability, and larger males are often more successful in monopolizing resources necessary for mating, either directly by monopolizing females or indirectly by nest sites. Sexual selection theory predicts strong selection for traits that increase reproductive success. Thus, larger males are often favoured by sexual selection (Bonduriansky and Rowe 2003; Bollache and Cezilly 2004; Dubey et al. 2009; Wacker et al. 2014).

Body size does not only vary within populations due to sexual selection favouring specific phenotypes but can also vary strongly among populations due to natural selection by the environment (Rundle et al. 2006). For example, it has been shown that body size is affected along latitudinal gradients and populations show either increasing (Bergmann's rule) or decreasing body size with increasing latitude (converse Bergmann's rule; Lindsey 1966; Murphy 1985; Blackburn et al. 1999; Stillwell et al. 2007). Body size variation among populations has also been found along other environmental gradients (e.g. temperature, precipitation, water depth; Hillebrand and Azovsky 2001; Smith and Brown 2002; Collins et al. 2005; Liao and Lu 2011). Thus, phenotypic variation in growth or absolute size may arise as a consequence of environmental conditions or due to sexual selection, and the effects may counteract one another. The interaction between natural and sexual selection may, therefore, vary among populations as a function of environmental heterogeneity.

The main objective of our study was to investigate how (1) quantity and quality of nesting resources, (2) demographic factors and (3) body size differ between populations of common gobies along a salinity gradient in the Baltic Sea and possibly affect (4) mating- and reproductive success. The Baltic Sea (Fig. 1) constitutes an extreme environment with a steep decrease in salinity from West (25 Practical Salinity Units = PSU) to East (1-2 PSU) and many species find their distribution limits across these salinity gradients (e.g. Jansen et al. 2009). In addition to spatial variation of salinity, surface water temperature along the coast of the Baltic Sea also shows a latitudinal and longitudinal gradient (HELCOM 1996). Hence, a seasonal surface water temperature gradient exists, with water temperatures rising later in the year in the North and East than in the South and West, which may affect for instance the duration of

breeding cycles, egg development (St Mary et al. 2004), and the strength of sexual selection itself (Monteiro and Lyons 2012).

Habitats in the Baltic Sea can differ greatly in nesting resource availability (Forsgren et al. 1996b), thus the cost of reproduction can differ between populations and can affect potential reproductive rates of both sexes (Ahnesjö et al. 2001). Salinity can not only directly act on fitness by affecting an organism's metabolism and population growth rate (Evans and Claiborne 2008) but also indirectly by limiting resources necessary for reproduction. Blue mussels (*Mytilus edulis*) or cockles (*Cerastoderma edule* and *C. glaucum*), which are frequently used by common goby males as nest substrate, are less tolerant to low salinity and either show a significant decrease in biomass from West to East, such as the blue mussel (Westerbom et al. 2002), or do not even extend into waters with salinities below 10-11 PSU, such as *Cerastoderma edule* (Brock 1980). For a better understanding of the strength and direction of sexual selection, it is necessary to know the degree of variation in nest availability between populations of Baltic common gobies. We predict a decrease in the overall nest availability and the occurrence of smaller, more fragile clams (*Mya arenaria*) with decreasing salinity from West to East in the Baltic Sea. This would imply consequences for the sexual selection regime among common goby populations along the salinity gradient, with higher competition over nests (i.e. intra-sexual competition) in the East than in the West and generally stronger sexual selection in low salinity populations.

Not only nest availability but also demographic factors like population density and sex ratios influence the degree and direction of competition in a population. Thus, we estimated population density as well as sex ratios (adult sex ratio: ASR and operational sex ratio: OSR) representing the ratio of adult individuals (ASR) respectively the ratio of ready-to-mate individuals (OSR) in a population, in five Baltic populations. Population density is expected to

be highest with high/intermediate salinity, which was shown for several marine fish species to increase growth rate and therefore reproductive success (Boeuf and Payan 2001). The operational sex ratio may be strongly affected by nest availability with for instance a shortage of nests implying that not all males of the population will find a nest for mating, and thus be female biased due to few nest holding males being ready-to-mate (Forsgren et al. 2004).

We measured body size of individuals along the salinity gradient as a possible phenotypic trait being directly affected by geographic variation in salinity. Due to the physiological stress of osmosis, we predict body size to decrease with decreasing salinity. Additionally, we conducted a mating assay to estimate mating- and reproductive success as well as differences in brood characteristics between populations. Variation in mating- and reproductive-success is expected to give a first assessment of differences in the sexual selection mode between populations. All data collected are from a field-based study conducted over several years (2012-2014) in order to provide new insights how geographic variation in nesting resources, population density, sex ratios and body size in a marine fish along an environmental gradient may shift fundamental aspects for mating- and reproductive-success and eventually cause local adaptations promoting population divergence. With the focus on linking spatially varying environmental factors to demographic and phenotypic differences between populations we aim to deliver a fundamental basis for further studies on environmental-dependent sexual selection.

Methods

Study species

The common goby (*Pomatoschistus microps*; Fig. 2) is a small euryhaline, benthic, annual fish species with a resource-based mating system. It reproduces repeatedly during a single

breeding season between May and August during which males compete over nest structures such as mussel shells, attract females by courtship displays and provide exclusive paternal care for the brood after spawning (Nyman 1953; Borg et al. 2002). Common gobies have a promiscuous mating system where males can care for eggs from several females simultaneously and females spawn with different males (Miller 1975). The common goby occurs on a wide range of conditions due to being the most temperature and salinity tolerant among the sand goby group (Fonds and van Buurt 1974) and occurs along the European Atlantic coast, at two populations in the Mediterranean and in the Baltic Sea, where it inhabits marine, brackish and extremely brackish habitats that exist within a relatively small geographic range (Fonds and van Buurt 1974).

Field study design

In a combined effort of habitat surveys and population sampling of five common goby populations along the salinity gradient (West to East) in the Baltic Sea (Fig. 1) with repeated visits early and late during the breeding season (Table 1), we collected data on: (1) salinity and temperature of the water multiple times using a HACH multi-probe (HACH Lange GmbH; Table 1), (2) quantity and quality of nesting resources, (3) population density and sex ratios (adult sex ratio (ASR) and operational sex ratio (OSR)), (4) body size of common gobies, and (5) variation in mating- and reproductive success as well as properties of broods.

Habitat survey

Populations were selected along the salinity gradient within the Baltic Sea from West to East (see Fig. 1). Each sampling site was visited between 2012 and 2014 two to five times both early and late in the breeding season (Table 1, sites marked with T). We defined the early breeding

season from the beginning of May to mid-June and the late breeding season from late June to late July. Habitat surveys were conducted by swimming two different transects during each visit at the five sites along a 20m lead line in shallow water (< 70 cm depth) colonized by common gobies. We collected data on nesting resource availability, population density and sex ratios within ca. 50 cm of each side of the lead line. Data collected along the two x 20m transects were pooled, because of very low sample sizes of variables at low salinity sites. For easier interpretation (in Results & Discussion) the selected sites were categorized depending on ICES and salinity (PSU; see Table 1) in: high (KR: 22.2), intermediate (KE: 16.8/VR: 20.0 and IP: 12.2) and low salinity habitats (GO: 7.2 and TV: 5.4). Nevertheless statistical analysis and graphical presentations are based on sites.

Nesting resources

The number of available nesting resources (including empty shells of *Mya arenaria*, *Mytilus edulis*, *Cerastoderma sp.*), as well as the number of natural nests occupied by males was counted along transects. To compare natural nest size variation between populations, we measured the diameter of randomly sampled *M. arenaria* shells used as nests (containing eggs). *M. arenaria* is the only mussel shell that occurs in low salinity and exhibits a size range that qualifies as potential nests for common gobies. Mussel nests were collected during the early breeding season, besides at IP where mussel nests were collected in the early and late season. Nests were collected in two consecutive years (2013-2014; exception: KE only 2014).

Chapter I

Table 1. Sampling sites in the Baltic Sea where data on habitat, population density and nest substrate were collected. Indicated are: sampling sites, geographic locations, ICES (International Council for the Exploration of the Sea) subdivisions, abbreviations for sites (Abb.), salinity (PSU) and salinity classes, mean surface water temperature (C°) pooled over three consecutive years (2012-2014) early and late during the breeding season and coordinates (Latitude and Longitude). Data collection of different variables vary between populations, years and seasons: T (transect data collected), S (body size measurements), asterisk (mating assay conducted). Blanks denote missing data.

Sampling site	Geographic location	ICES	Abb.	Salinity PSU	Salinity class	Temp. C°		Latitude	Longitude	2012		2013		2014	
						early	late			early	late	early	late	early	late
Kristineberg	western Sweden	23	KR	22	high	16.1	19	58°24'N	11°46'E	T/S*		T/S	T	T/S	
Vrinnars	East Jutland, Denmark	22	VR	20	intermediate	---	20	50°14'N	10°30'E		*				
Kerteminde	Funen, Denmark	22	KE	17	intermediate	18.1	19	55°44'N	10°65'E		T/S*			T/S	
Island Poel	Bay of Mecklenburg, Germany	22	IP	12	intermediate	15.1	18	53°99'N	11°48'E		T/S*	T/S	T/S	T/S	T
Gotland	Gotland Basin, Sweden	28	GO	7	low	16.6	18	57°78'N	18°94'E	T/S*		T/S	T/S	S	T
Tvärminne	Southern Finland	29	TV	5	low	13.5	15	59°82'N	23°14'E	T/S*		T/S	T/S	T	T/S

Population density and sex ratios

Our data on individual counts (population density) along transects include females of all three ripeness stages (R1-R3 explanation see below; Fig. 3) and free swimming males, not sitting inside a nest. Individual male and female count data can be used to calculate the ASR (calculated as the fraction of adult males to all adult individuals (Wilson et al. 2002), because typically only adult individuals are present from May-July. To estimate the operational sex ratio (OSR) males occupying a nest were categorised as males ready-to-mate and females were categorised into three ripeness stages (R1-R3) according to the roundness of their bellies. Females ripeness stages are easily distinguishable by visual inspection: R1 females do not show any extended bellies (no ripe eggs), R2 females show a rounded belly (some ripe eggs), while R3 females show often a bright orange-pinkish extremely round belly (belly filled with ripe eggs; see Fig. 3). To calculate the OSR (fraction of mating-ready males to all mating-ready individuals; Kvarnemo and Ahnesjö 1996) only females of R2 and R3 were included.

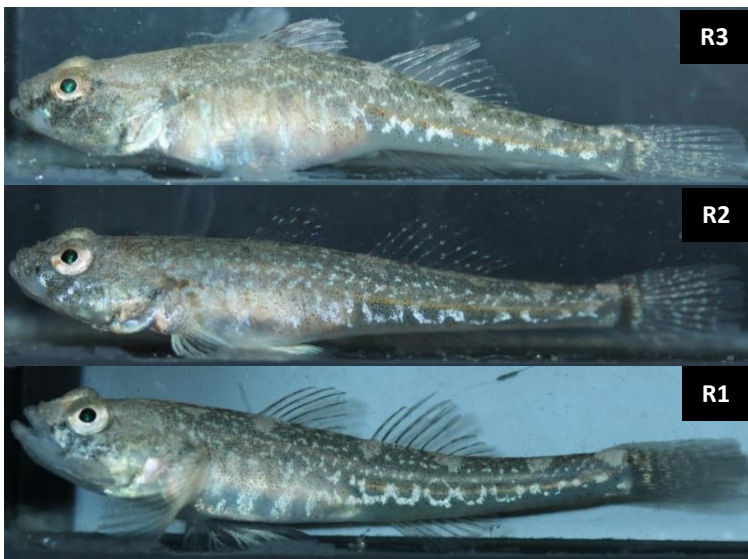


Figure 3. Female common gobies (*Pomatoschistus microps*) of three ripeness stages (R1- R3): R1: female does not show an extended belly (no ripe eggs), R2: female shows a rounded belly (some ripe eggs) and R3: female with an orange-pinkish extremely round belly (belly filled with ripe eggs).

Body size

Fish of both sexes (Fig. 2) were caught in shallow waters near the coast at five sampling sites using hand trawls (always the first 25 fish caught were used for analysis) for body size measurements (Table 1, sites marked with S). We measured total length (TL) to the nearest 1 mm. Whenever possible measurements of 25 individuals of each sex were used (with some exceptions in 2013: GO early: females: N = 20, males: N = 24, GO late: females: N = 6; TV early: males: N = 21 and late: N = 18). After measurements were taken, fish were released back to their natural habitat.

Mating assay

In order to gather data on (a) nest colonisation, (b) mating success, (c) reproductive success and (d) properties of broods, an assay with standardised nest availability, exposure (72h) and quality was carried out in 2012 at five different sites in the Baltic Sea (KR, VR, IP, GO and TV; for details see Table 1 sites marked with *). Because of logistic reasons, the field assay was not conducted at KE but instead at VR, a population ca. 80km further north, with similar ecological conditions and habitat structure but slightly higher salinity (20.0 PSU; for details see Table 1 and Fig. 1). Thirty ceramic tiles (4x4 cm), readily accepted as nesting resource by common goby males, were put out in shallow water (< 70 cm depth). After 72h we checked each nest for nest occupation by a resident male and collected all tiles and photographed broods if the male received eggs. Male reproductive success was estimated by the brood size (mm²), which was measured using ImageJ version 1.47 (Rasband 1997-2015; Schneider et al. 2012). Even though it is common for males to cannibalise on eggs, due to visible residues of mucus it was possible to see where eggs had been attached to the tile prior to cannibalism (authors' pers. obs). This method thus allowed us to measure the initial brood size, even when

at the time of nest retrieval some eggs had been cannibalised. To make sure that our measure of reproductive success was not influenced by differences of the density or size of the eggs, the number of eggs within a 0.5 x 0.5 cm² square were counted as well as the size of these counted eggs were measured using ImageJ (number of broods: KR, VR, GO: N = 4 and IP, TV: N = 3). Since only one male received eggs in TV after 72h, this population was excluded from analyses of reproductive success. Measures of egg density and egg size for TV, however, were collected from two artificial nests that were exposed for 92h.

Data analyses

Statistical procedures

Data on mating resources, population density, sex ratios, body size and reproductive success as well as brood characteristics collected over consecutive years (2012-2014) were centred around the yearly mean to account for differences between years. Normality of each variable tested was checked via visual inspection of residuals and q-q normality plots. To achieve normality, count data were log-transformed (log₁₀; 'Available nests') and proportion data were 'Arcsine' transformed (asn; 'Proportion of occupied nests' and 'Sex ratios') using the 'Anscombe' variant

$$y = \arcsine \sqrt{\frac{(n \text{ success} + \frac{3}{8})}{(n \text{ total} + \frac{3}{4})}} \text{ (Anscombe 1948)}$$

suggested by (Zar 1984) prior to centring around the yearly mean.

We run for each variable independently a linear model (LMs) in R using R Studio version 3.03 (R Core Team, 2012) with centred data as outcome variable and '*populations*' set as factors ranked according to salinity (from West: high to East: low) as well as '*season*' (early and late)

as independent variables. Model selection was conducted and non-significant interactions (*'population:season'*) were excluded from the model. If a significant interaction with *'season'* was found, the data set was divided into 'early' and 'late' and analysed for both seasons separately. Pairwise post-hoc comparisons between populations were conducted using a Bonferroni or Benjamini Hochberg method controlling for the false discovery rate (at level α).

Male body size and M. arenaria shell length

The mean of mussel shell length [mm TL] of *Mya arenaria* nests and male body size [mm TL] for each sampling event (N = 9) were correlated using a Spearman's rank correlation rho (ρ) in R. Only mussel shells and corresponding average male size collected in the same year and same season were correlated.

Mating assay

For the binomial response variables in the mating assay on nest occupation and mating success after 72h of each artificial nest (N = 30) frequencies of occurrence (frequency of success and failure) were used. To test if observed values differed from expected values (based on either the average proportion of nest occupation across all populations or, for mating success, on the population-specific proportion of nest occupation) we used contingency table analyses with Pearson Chi-square (χ^2) and log-likelihood ratio tests with Yates correction in SPSS version 22 (IBM SPSS, Statistics).

Results

Nesting resources

Nest quantity

Low salinity sites (GO and TV) showed a much lower number of available nests than high (KR) or intermediate (KE, IP) salinity sites (Fig. 4a). The availability of natural nests (*Mya arenaria*, *Mytilus edulis*, *Cerastoderma edule*, *C. glaucum*) along the salinity gradient differed significantly between populations (but not between seasons; Table 2).

Proportion of occupied nests

The intermediate site IP, with the highest number of nests available, showed the overall lowest proportion of mussel nests taken up by males (Fig. 4b). The proportion of occupied natural nests at the low salinity sites (GO and TV) showed a trend to be highest, but did not differ significantly from the intermediate site KE (Fig. 4b). The site with the highest salinity (KR) showed a significantly lower proportion of occupied nests than the site with the lowest salinity (TV). The proportion of occupied natural nests differed significantly between populations (but not between seasons; Table 2).

*Quantity and quality of soft-shell clams (*M. arenaria*)*

The availability of *M. arenaria* was significantly higher in high and intermediate salinity sites than in low salinity sites (Table 2, Fig. 4c). The size distribution of *M. arenaria* showed a clear separation between the two highest (KR and KE, laying East of the Baltic entrance, see Fig. 1) and three lower salinity sites (IP, GO and TV, laying West of the Baltic entrance, see Fig. 1; Table 2, Fig. 4d).

Chapter I

Table 2. Results of final linear models (LMs) for all variables tested. Given are groups, the specific variables tested and if variables were transformed (trans. log (log₁₀) or ans (arcsine)) to achieve normality; the variation that is explained by the model in % (adjusted R²), the fit of the final model; the F-statistic of the independent variables ‘population’ (pop) and ‘season’ and the F-statistic of the interaction term. Significant results are denoted in bold with ** (P < 0.0001) and * (P < 0.05). Non-significant results are denoted with NS, empty rows denote ‘not tested’.

Group	Variable	Variation		Final model	pop		season		sea*pop		
		trans.	adj. R ² (%)	df	F	df	F	df	F	df	F
Mating resources	Available nests	log	77	5, 35	28.2**	4,35	34.9**	1, 35	1.1	NS	NS
	Proportion of occupied nests	asn	46	5, 35	7.8**	4, 35	9.4**	1, 35	1.5	NS	NS
	Soft-shell clams		37	4, 36	6.9**	4, 36	6.9**				
	Size soft-shell clams		60	4, 83	33.5**	4, 83	33.5**				
Body size	Female		32	5, 294	28.6**	4, 294	22.3**	1, 294	53.6**	NS	NS
	Male		27	5, 294	22.8**	4, 294	28.0**	1, 294	1.9	NS	NS
Densities & Sex ratios	Number of females		70	5, 35	20.2**	4, 35	23.5**	1, 35	6.6*	NS	NS
	Number of males		69	5, 35	18.6**	4, 35	22.4**	1, 35	3.6	NS	NS
	ASR	asn	20	5, 35	3.0*	4,35	3.5*	1, 35	0.9	NS	NS
	Number of ready-to mate females		70	5, 35	19.8**	4, 35	23.3**	1, 35	5.9*	NS	NS
	Number of ready-to mate males		50	9, 31	5.5*	4, 31	9.0**	1, 31	0.9	4, 31	3.1*
	OSR	asn	37	5, 35	5.6*	4,35	6.0*	1, 35	3.9	NS	NS
Mating assay	Brood size		18	4, 44	3.7*	4, 44	3.7*				
	Egg size		64	4, 761	338.1**	4, 761	338.1**				
	Egg density		42	4, 13	4.1*	4, 13	4.2*				

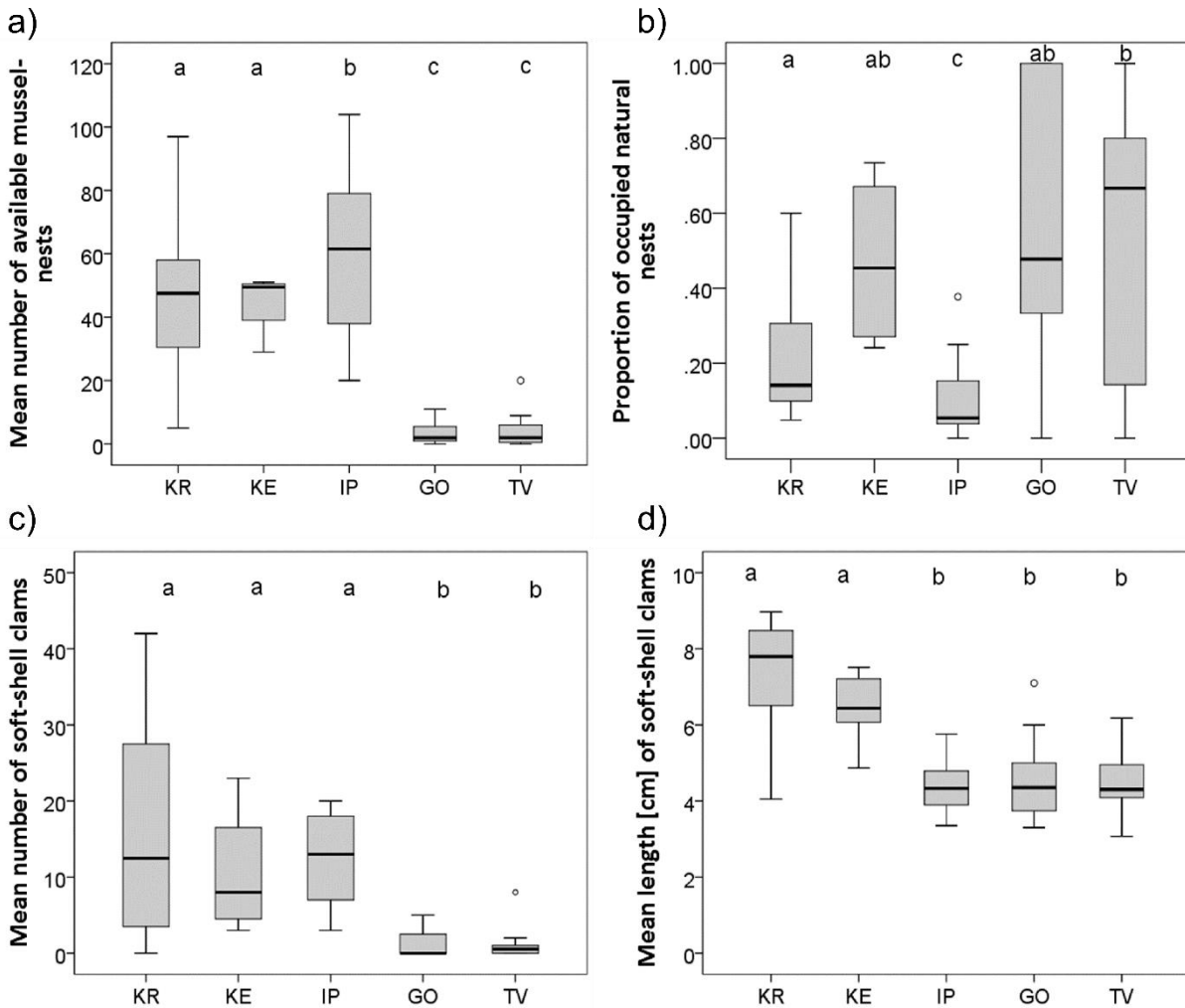


Figure 4. Data collected along transects (2x20m) between 2012-2014 on a) mean number of available mussel-nests (free and occupied), b) the proportion of natural mussel-nests occupied and c) the mean number of soft-shell clams (*M. arenaria*) as well as their d) mean length [cm]. Box plots represent the medians and the first and third quartiles. Whiskers represent the most extreme data point ≤ 1.5 times the interquartile range from the box. Outliers are shown as separate data points. Letters above box plots indicate significant ($P < 0.05$) differences between populations whereby shared letters denote categories not significantly different in multiple comparisons. The total number of transects between populations varies, for details and site abbreviations see Table 1. Note that for easier interpretation, untransformed non-standardized data is shown. Significances refer to centred (a, c, d) and transformed (a (\log_{10}), b (asn)) data (see also Table 2).

Population density

The number of males and females counted along transects differed between populations (Table 2) and was highest at the intermediate sites, and especially so in KE (mean number of individuals \pm SE: KE: males: 82.5 ± 10.9 and females: 83.3 ± 16.1 compared to males: 11.3 ± 2.2 and females: 10.2 ± 2.8 from KE, IP, GO, TV; Fig. 5a). Season also had an effect on population density (Table 2), which was overall higher in the early season (males: 22.3 ± 6.0 , females: 23.8 ± 8.3) than in the late season (males: 14.7 ± 5.4 , females: 11.7 ± 4.2). We also found significant differences in population density within populations between seasons (for females in: KE: $F_{1,31} = 8.3$, $P = 0.007$ and IP: $F_{1,31} = 9.3$, $P = 0.005$; for males only in: IP: $F_{1,31} = 4.7$, $P = 0.038$; Table 2).

Operational sex ratio

The OSR was significantly different between populations (Table 2). The low salinity site GO was significantly more male biased than all other populations except KR (Fig. 5b). The number of ready-to-mate males showed a significant interaction between population and season (variation among populations early: 53%, $F_{4,14} = 6.0$, $P = 0.005$; late: 38%, $F_{4,17} = 4.2$, $P = 0.016$). More ready-to-mate males were counted early than late in the season at the two sites with the highest salinity (mean number of ready-to-mate males \pm SE: KR: early: 13.5 ± 8.9 , late: 5.5 ± 1.4 ; KE: early: 33.5 ± 2.5 , late: 11.0 ± 4.0), which were also the two sites showing the overall highest number of ready-to-mate males. At the intermediate site IP (early: 2.0 ± 1.2 , late: 7.8 ± 2.5) and the low salinity site GO (early: 1.7 ± 1.2 , late: 2.0 ± 1.1), however, more ready-to-mate males were counted in late season than in early season. For ready-to-mate females no significant interaction between population and season was detected (Table 2).

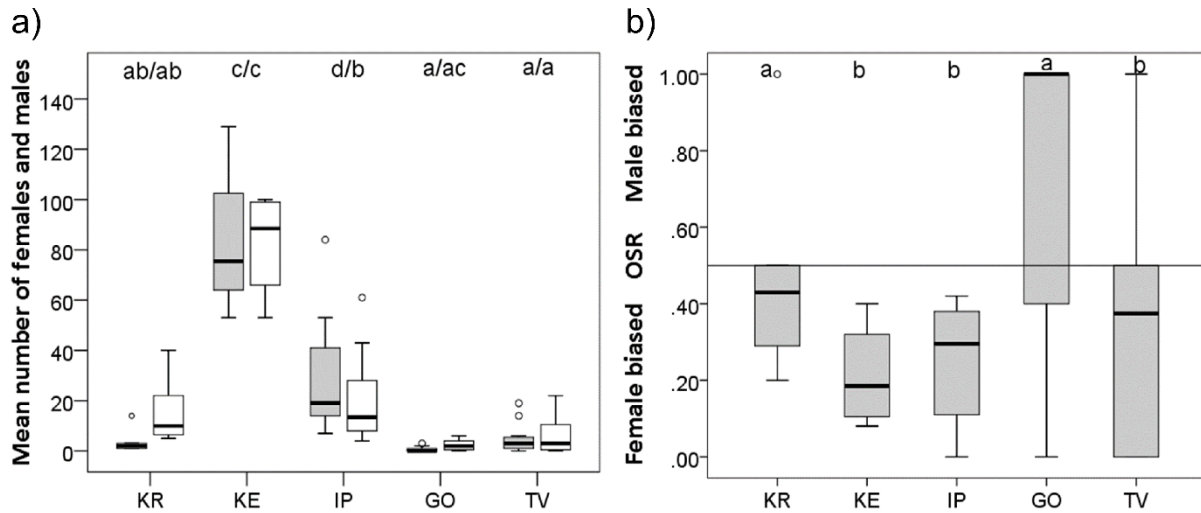


Figure 5. Transect (2x20m) data (years 2012-2014) on a) female (grey boxes) and male (white boxes) common gobies counted (population density) and b) the operational sex ratio (OSR = the fraction of all ready-to mate males to all ready-to mate individuals). See Table 1 for details for site abbreviations and Fig. 4 for details on box plot graphs.

Body size

Males as well as females showed a significant decrease in body size with decreasing salinity from West to East (Table 2, Fig. 6a, b). Females of the high salinity site KR (mean body size in mm \pm SE: KR: 39.8 ± 0.30) were 1.5 mm longer than females of the intermediate sites KE and IP (38.3 ± 0.27) and 3.4 mm longer than females of the low salinity sites GO and TV (36.4 ± 0.19). Males of the high salinity site KR (39.5 ± 0.28) were 2.5 mm longer than males of the intermediate sites KE and IP (37.0 ± 0.27) and even 4.9 mm longer than males of the low salinity sites GO and TV (34.6 ± 0.20).

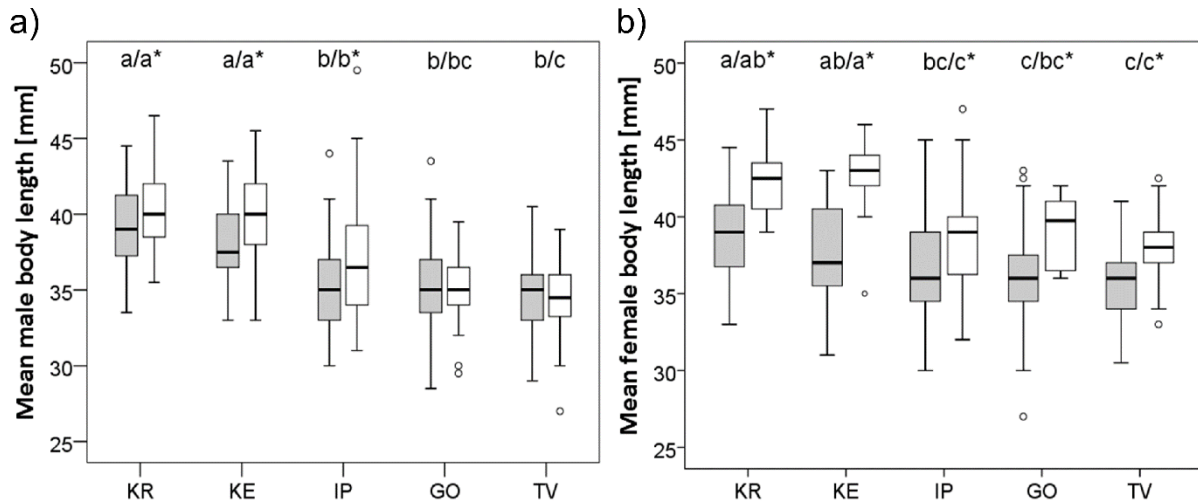


Figure 6. Mean centred body length [TL mm] for a) males and b) females measured early (grey boxes) and late (empty boxes) during the breeding season over three consecutive years (2012-2014). Asterisks denote significant differences between early and late season within populations. See Table 1 for site abbreviations and Fig. 4 for details on box plot graphs.

We found a relation between season and body size, wherein particular females were found to be larger late in the season (Table 2). Over all populations showing seasonal growth (for males only populations KR, KE, IP), females were about 3mm longer late in the season (early: 36.9 ± 0.18 , late: 39.9 ± 0.23), while males only grew 0.6 mm (early: 37.6 ± 0.27 , late: 38.2 ± 0.33). Males of low salinity sites GO and TV did not grow over the season (Table 2, Fig. 6b).

Male body size and size of *Mya arenaria*

Mean male body size showed a strong positive correlation with the size of *M. arenaria* mussels used as nests (Spearman's rank correlation: $\rho = 0.85$, $P = 0.006$; Fig.7). The high salinity site KR showed the largest *M. arenarias* as well as the largest males. Following the salinity gradient both shell and male sizes decreased. The site with the lowest salinity (TV) showed also the smallest mussel nests as well as males. Natural nests with *M. arenaria* shells as nest substrate

were at the high salinity site KR about 30 mm larger (mean *M. arenaria* length in mm \pm SE: 74.4 ± 4.7) than at the low salinity site TV (44.5 ± 1.9 ; Fig. 7).

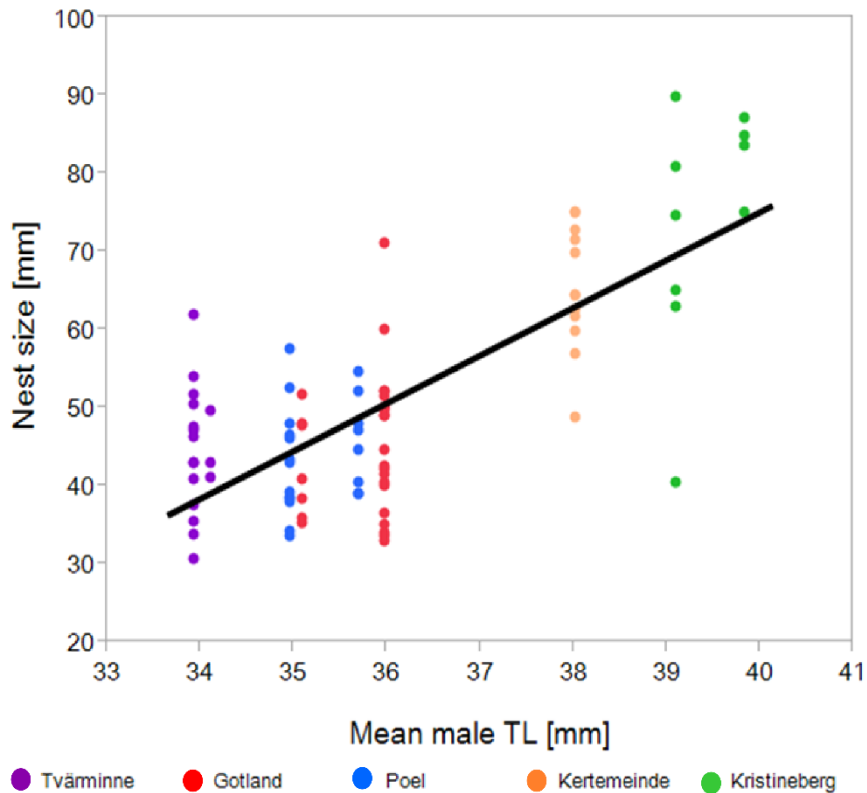


Figure 7. The size [mm] of mussel nests *Mya arenaria* (*Mya*) correlated with male mean body size [mm TL] of each site sampled in the corresponding year and season (black line indicates linear regression fit). Nest clams from the high salinity site KR (green), the intermediate salinity sites KE (orange) and IP (blue) and the low salinity sites GO (red) and TV (pink) were collected during the early breeding season. Mussel nests at IP were also collected late during the breeding season. Mussels at all sites were collected in 2013 and 2014 except IP and KE, which were only sampled in 2014.

Mating assay

Nest occupation

The nest occupation rate of the 30 artificial nests after 72h experimental exposure varied significantly between the five populations (log-likelihood-test with Yates correction: $\chi^2 = 47.8$,

$df = 4$, $P < 0.0001$, $N = 150$; Fig. 8a, grey bars). Testing against the overall average occupation rate across all populations, revealed that the number of occupied nests at the high (KR: $\chi^2 = 0.5$, $df = 1$, $P = 0.487$, $N = 12$), the intermediate site VR ($\chi^2 = 3.2$, $df = 1$, $P = 0.073$, $N = 9$) as well as at the low salinity sites TV ($\chi^2 = 0.6$, $df = 1$, $P = 0.443$, $N = 17$) did not significantly differ from the expected number of males occupying nests. The intermediate site IP however, showed a significantly lower nest occupation rate than expected ($\chi^2 = 10.6$, $df = 1$, $P = 0.001$, $N = 5$), while the low salinity site GO showed a significantly higher rate of occupied nests than expected ($\chi^2 = 26.6$, $df = 1$, $P < 0.0001$, $N = 29$).

Mating success

Not all males that had taken up a nest were successful in also receiving eggs within 72h and a significant difference in the frequency of mated nest holders was detected between populations (log-likelihood-test with Yates correction: $\chi^2 = 44.5$, $df = 4$, $P < 0.0001$, $N = 72$; Fig. 8a, white bars). Results of chi-square tests revealed that the number of mated nest holders did not differ significantly from expected numbers based on nest occupation at the high salinity site KR ($\chi^2 = 0.04$, $df = 1$, $P = 0.852$, $N = 9$) nor at intermediate sites (VR: $\chi^2 = 0.08$, $df = 1$, $P = 0.775$, $N = 7$; IP: $\chi^2 = 0.009$, $df = 1$, $P = 0.924$, $N = 3$). For the two low salinity sites we found opposing results; while there were significantly more mated nest holders than expected at GO ($\chi^2 = 12.3$, $df = 1$, $P = 0.0005$, $N = 29$), there were significantly less mated nest holders than expected at TV ($\chi^2 = 27.7$, $df = 1$, $P < 0.0001$, $N = 1$).

Reproductive success

Brood size (representing male reproductive success) was significantly different between populations (Table 2), with males at the intermediate site IP showing significantly higher reproductive success (mean brood area in $\text{mm}^2 \pm \text{SE}$: 93 ± 120.8) than males of all other

populations (Fig. 8b). Broods of all other populations did not significantly differ in size (KR: 648 ± 66.9 , VR: 597 ± 60.0 , GO: 581 ± 32.6 ; Fig. 8b). Filial cannibalism on broods was detected in three out of five populations. The highest rate of filial cannibalism was detected at the high salinity site KR where overall eight out of nine broods showed signs of cannibalism, of which five broods were completely cannibalised after 72h during the mating assay. At the intermediate site VR three out of seven broods showed signs of cannibalism with one brood being completely cannibalised. At the low salinity site GO only three out of 29 broods showed mild signs of cannibalism. At IP (and TV: $N = 1$) no cannibalism was detected.

Brood characteristics

Egg density

Egg density was significantly different between populations (Table 2, Fig. 8c) with males of the low salinity site GO having significantly fewer eggs within 0.5×0.5 cm (mean egg number \pm SE: GO: 33.7 ± 1.1) than males of high and intermediate sites (KR: 43.3 ± 3.4 , VR: 48.2 ± 1.9 , IP: 48.0 ± 3.8). There was no significant difference between high, intermediate and the low salinity site TV (TV: 41.3 ± 0.9 ; Fig. 8c).

Egg size

Egg size data suggest males of intermediate salinity sites (VR and IP) to receive the smallest eggs. Egg size differed significantly between all populations, except between the high (KR) and the low (GO) salinity sites, where broods contained the biggest eggs (mean egg size in $\text{mm}^2 \pm$ SE: KR: 0.465 ± 0.01 , GO: 0.474 ± 0.02 ; Table 2; Fig. 8d). At the intermediate sites VR and IP as well as the low salinity site TV males received significantly smaller eggs (VR: 0.267 ± 0.01 , IP: 0.305 ± 0.01 , TV: 0.396 ± 0.02 ; Fig. 8d).

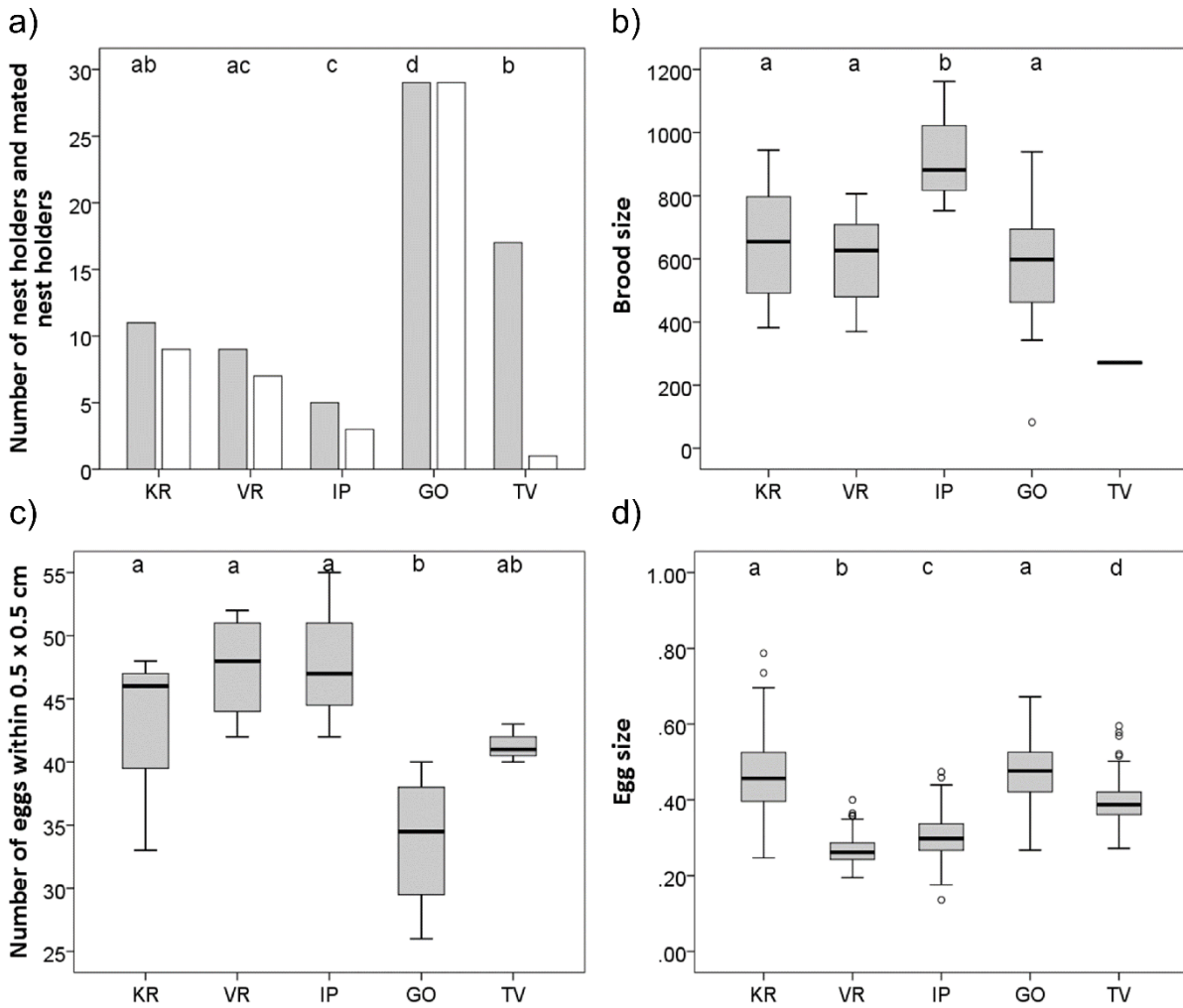


Figure 8. Results of the mating assay after exposing 30 artificial nests for 72h at five different sites in the field. a) number of nests occupied by nest holders (grey boxes; letters indicate significant differences between populations) and mated nest holders (empty boxes); b) mean brood size (mm²) of mated nest holders; c) 'egg density' (number of eggs within 0.5 x 0.5 cm); d) 'egg size' (mm²). See Table 1 for site abbreviations and Fig. 4 for details on box plot graphs.

Discussion

We found that geographic variation influences fundamental aspects of mating success in common gobies, which may directly or indirectly affect the sexual selection regime of common goby populations along the salinity gradient in the Baltic Sea. Transect data revealed that quantity and quality of nesting resources (*M. arenaria*) generally decreased with decreasing salinity from West to East and so did the body size of common gobies.

Common gobies have a resource-based mating system, where the availability of mussel shells is crucial for successful mating and reproduction. Because mussels are marine species, low salinity waters pose suboptimal habitat conditions (Kube et al. 1996). One of only few mussel species that occurs throughout the full salinity gradient at all five sampled sites is the soft-shell clam (*M. arenaria*). At low salinity sites it is also the only one of sufficient size to serve as nest resource for *P. microps*. As expected, we found a considerable decrease in the density of *M. arenaria* in salinities of 7 PSU and lower. Interestingly, it seems that the threshold for growth of *M. arenaria* is roughly around 15 PSU (according to our measurements between 19-13 PSU), because despite its high density at IP (12 PSU), its size was comparable to that of the shells measured at the low salinity sites GO (7 PSU) and TV (5 PSU). Our findings support those from Matthiessen (1960), who showed that *M. arenaria* can survive in salinities as low as 4 PSU; however, the feeding rate was already negatively affected below 15 PSU, as the pumping rate is then significantly reduced. While KR and KE lay within the Kattegat, IP is just west of the line of Falsterbo (south Sweden) and Travemünde (Germany), marking the entrance of the Baltic Sea proper (see Fig. 1). Thus IP, GO and TV may represent in certain aspects the conditions of the Baltic Sea better than KR and KE.

Differences in body size were already found in a previous study on the closely related sand goby (*P. minutus*), which compared the mode of sexual selection between the high nest

availability site (KR) and the low nest availability site (TV; Forsgren et al. 1996b). Males of KR (high salinity) were also found to be larger than males of TV (low salinity), however, no clear conclusion about male size differences was drawn and no link to differences in salinity between high and low nest availability sites were hypothesized as a possible explanation. With our extended study on common gobies, incorporating a total of five populations spanning the entire salinity gradient we fill the gap of knowledge on how body sizes vary between high and low salinity sites. Results show a linear decrease of body size in common gobies along the salinity gradient in the Baltic Sea from west (KR) to east (TV). The common goby is a marine species, which originated from the Mediterranean Sea (Simonovic 1999) and must cope with low salinity conditions, which likely constitutes physiological stress. Fish with high metabolic costs of osmoregulation often compensate energy allocation to growth with 20 to > 50% of the total fish energy budget being dedicated to osmoregulation (reviewed in Boeuf and Payan 2001). This could explain a decrease in body size of *P. microps* with decreasing salinity and increasing costs for osmoregulation (Boeuf and Payan 2001; Glover et al. 2012; Passow et al. 2015). Therefore, our findings that fish body size as well as shell size (and shell density) decrease with decreasing salinity could be explained by both species originating from a fully marine background being affected in a similar way by low salinity conditions.

Another explanation for the habitat-specific size differences in common gobies might be the decrease in shell size as a result of the decrease in salinity. It is possible that small common goby males (as well as females) of low salinity habitats facing small nests had over time an evolutionary advantage by actually being able to fit inside small nests. Size-assortative nest choice has been shown for *P. microps* (Magnhagen and Vestergaard 1993) and many other fish species with a resource-based mating system and paternal care (Cote and Hunte 1989; Kvarnemo 1995; Natsumeda 1998; Lehtonen and Lindström 2004; Takegaki et al. 2008). Even

though there is evidence from a variety of fish species, including *P. microps* (Lindström 1988; Hastings 1992; Magnhagen and Vestergaard 1993), that larger nests generally contain more eggs resulting in higher reproductive success, there seems to be a trade-off between maximizing surface area for egg deposition and minimizing costs of nest maintenance and defence. For common goby males in a low salinity habitat a choice between large and small nests however is rare anyway. It is likely that small males are under stronger sexual selection in low salinity habitats leading over time to overall smaller common gobies inhabiting low salinity habitats compared to larger males inhabiting high salinity habitats with large nests. The strong positive correlation between male size and mussel size highlights the effect salinity can have on species metabolism and therefore growth rate, and suggests how natural selection and sexual selection can be linked.

Why did common gobies at all colonize the eastern parts of the Baltic Sea if adverse conditions caused a decrease in nest quality and quantity, and a reduction in growth rate? One explanation may be that high population densities can result in lower fitness for individuals that settled originally in the best possible habitat (likely to be: high/intermediate salinity, western Baltic Sea; Boeuf and Payan 2001). Thus, if a population density would be reached at which expected fitness in a poorer habitat would be as high as in the best habitat, colonisation of the poorer habitat (low salinity, eastern Baltic Sea) may begin (Fretwell 1972). Our results on population density generally support this theory, showing low population densities in low salinity habitats (GO and TV) and the highest population densities at intermediate sites (KE and IP). According to this scenario, however, one would expect to find the highest population density at the high salinity site (KR), yet population density at KR was almost as low as at TV, the site with the lowest salinity. One very likely explanation for this observation are sea level fluctuations around KR due to deep low pressure passages over the Bothnian Bay, combined

with high pressure over the southern Baltic (SMHI, Swedish Meteorological and Hydrological Institute). During the sampling period in KR sea level was below normal (early season 2013/2014: -29mm/-258 mm, late season 2013: -128 mm), which could have led to common gobies staying in deeper waters rather than start breeding in shallow, unpredictable coastal areas. We, therefore, recommend to treat results on population density in KR with caution, due to unusual meteorological and hydrological abnormalities during sampling. Alternatively, intermediate salinity levels might constitute optimal habitat conditions due to high nesting resource availability and intermediate abiotic factors indicated by high population density (Gilliers et al. 2006).

Furthermore, population densities differed not only between populations but also over the season. Generally, more fish were counted early than late in the breeding season. Common gobies are annual fish and it was shown for the closely related sand goby that males facing intra-sexual competition died earlier than males not competing, because of increased stress levels and energy depletion (Lindström 2001). A drop in population size as the breeding season progresses might be caused by a depletion of energy reserves after reproduction leading to high mortality of this annual fish.

The OSR was significantly more male biased in the low salinity site GO and showed a trend for a male bias for the other low salinity site TV as well as the high salinity site KR, while intermediate salinity sites were clearly female biased (Fig. 5b). These findings however, are correlated with the overall very low population density at the low and high salinity sites, making it more likely to count stationary males sitting in their nests (defined as ready-to-mate), than counting free swimming ready-to mate females. No differences in the OSR were found between seasons. This result contrasts those of Forsgren et al. (2004), who found in a closely related goby species (*Gobiusculus flavescens*) inhabiting the high salinity sites in the

Baltic Sea that late in the season more females were ready-to-mate than males, and thus a shift in the OSR. Nevertheless, more frequent sampling could detect subtle differences between seasons and reflect the whole progress of one breeding season more accurately. On the other hand, such a clear temporal shift in the OSR may not exist in *P. microps*. Overall, we are cautious with our interpretation of OSR results because of small sample sizes (KR, GO, TV) and therefore larger variation between sites.

Results of the mating assays corresponded well with data on natural nests collected along transects. The nest occupation rate of artificial nests mirrored natural nest availability; low salinity sites showed the highest artificial nest occupation rate (natural nest availability: low), followed by KR and KE (natural nest availability: intermediate), and IP (natural nest availability: high). These findings are similar to results on artificial nest occupation rate of a field study in KR and TV found in sand gobies where males of TV occupied more nests and did so faster than males of KR (Forsgren et al. 1996b; see also Borg et al. 2002).

The frequencies of the mating success by males occupying an artificial nest were, however, unexpected. While all nest holding males of the low salinity site GO received eggs, this was true for only one of the nest holding males at the other low salinity site TV. A plausible explanation is lacking here, because both GO and TV showed a rather male-biased OSR. However, the conducted mating assay represents a single, short time frame (72h) only, during which abiotic factors like unstable weather conditions may cause females to reduce spawning (authors pers. obs.). In fact, during the period the mating assay was carried out in 2012, water temperatures at all sites ranged between 15°C and 20°C, but did not exceed 14°C in TV. The rise of water temperatures during the breeding season of *P. microps* starts later in the North and East, resulting in spatial-temporal variation in water temperature in the Baltic Sea. Water temperature plays a crucial role in female egg development as well as in the duration the eggs

need to hatch, and therefore affects the reproductive cycle of both sexes as well as the operational sex ratio (Pauly and Pullin 1988; Kvarnemo and Ahnesjö 1996; Ahnesjö et al. 2001).

The highest reproductive success (brood-size measured) was found at the intermediate population IP, which at the same time was also the population with the lowest artificial nest occupation rate. Other studies suggested that females of various fish species with paternal care prefer to lay eggs in nests, which already contain eggs (Ridley and Rechten 1981; Jamieson 1995; Forsgren et al. 1996a; Goulet 1998). This might explain why only 60% of nest holding males received eggs at IP, but all of these males were guarding large broods (suggesting clutches of two or more females). Brood-size of KR, VR and GO were similar in size. By definition is variation in mating- and reproductive-success determining the strength and direction of sexual selection within populations as well as between populations (Howard 1983).

Interestingly, although brood-size of males at the high salinity site KR and the low salinity site GO were similar, the egg density was higher at KR than at GO. Broods of intermediate sites (VR and IP) contained the smallest eggs at high density and broods of the low salinity site TV contained eggs of intermediate size and density. Our results on differences between populations in egg size and egg density highlight that inappropriate techniques chosen to estimate reproductive success can lead to false conclusions.

Why are the eggs at GO as large as those at KR but differ in density? Generally, in marine teleost fish, egg size variation appears to be related to maternal effects (Chambers and Leggett 1996), which would imply that females of KR (high salinity, large females) should produce larger eggs than females of GO (low salinity, small females). On the other hand, externally developing eggs are in constant contact with the surrounding water, which can contain

pathogens like water molds (*Saprolegnia*) affecting egg viability. High salinity decreases the infection rate of eggs, while high density of eggs increases infection rate (Lehtonen and Kvarnemo 2015a). Therefore, egg density at the low salinity sites is low (GO) to intermediate (TV), which might represent an evolutionarily effective strategy to reduce the egg mold spreading risk in low salinity habitats. Laying eggs at low density means that females cannot lay as many eggs in one nest as when they would lay eggs very densely. In addition, females of low salinity sites are smaller than those of high salinity sites, and body size is directly correlated to fecundity in many fish species (Koops et al. 2004). To compensate these trade-offs it is likely that females of low salinity sites invest in egg quality (size) rather than in egg quantity, because large eggs imply larger new-borns, which increases their survival rate especially during the first critical days when larvae still nourish from their yolk sack (Tamada and Iwata 2005; Allen et al. 2008).

Conclusion

Our results suggest that low salinity sites in the Baltic Sea rather pose a suboptimal habitat choice for common gobies. Mussel shells necessary for reproduction decrease in quality and quantity, and the population density and growth rate of *P. microps* itself is reduced, suggesting that geographic variation of abiotic and biotic factors can strongly influence populations' life history and prospects for mating. We demonstrated the importance of considering environmental parameters, such as resource availability among populations, in studies investigating a species' sexual selection regime. In particular environmental gradients seem to promote a basis for environmental-dependent sexual selection; neglecting differences in abiotic factors between populations may lead to false conclusions about sex role dynamics and the overall outcome of sexual selection. We encourage standardized common garden

experiments to empirically test the effect of salinity on physiological traits such as body size, which would allow to evaluate the relative degree of phenotypic plasticity. To evaluate if Baltic common gobies are adapted to salinity due to natural selection or due to drift population genetic analyses are required (see Chapter II).

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CHAPTER II

Population Divergence in Common Gobies: Neutral or Adaptive Evolution?

I.M. Mück, K. B. Mobley, P. Gienapp, K.U. Heubel

Abstract

Population divergence at neutral genetic markers is caused by genetic drift due to reduced gene flow, which can arise by isolation by distance (IBD; neutral evolution) and/or isolation by environment (IBE; adaptive evolution). Here, we investigate the relative contribution of IBD and IBE driving population divergence in common gobies (*Pomatoschistus microps*). We used microsatellite markers to investigate genetic population differentiation of common gobies focusing on the Baltic Sea but including Mediterranean, the Atlantic and North Sea populations as benchmarks. Additionally, we investigated if phenotypic variation in body shape of Baltic common gobies is caused by natural selection or genetic drift by comparing phenotypic differentiation (P_{ST}) with neutral genetic differentiation (F_{ST}). We found a clear genetic separation between Mediterranean, Atlantic, North Sea (high salinity) and Baltic Sea populations (mid to low salinity). Furthermore, eastern Baltic populations (low salinity) form a monophyletic group within the Baltic cluster. Generalized Dissimilarity Models (GDMs) suggest IBE due to salinity to be as likely an explanation as IBD to drive population differentiation in common gobies. Phenotypic differentiation (P_{ST}) of body shape was found to be greater than neutral genetic differentiation (F_{ST}) among populations suggesting that divergent selection is favouring different phenotypes in *P. microps* in the Baltic Sea. This study demonstrates that both IBE and IBD are important processes driving divergence in this species.

Introduction

It has become clear through theoretical (Wright 1943; Kimura 1984; Shafer and Wolf 2013; Sexton et al. 2014; Wang and Bradburd 2014) and empirical studies (Via et al. 2000; Pogson et al. 2001; Crispo et al. 2006; Cooke et al. 2012) that both neutral as well as adaptive evolutionary processes can lead to population divergence. However, their relative contribution to population differentiation is often poorly understood. Neutral evolution arises through genetic drift and is enhanced by limited dispersal due to isolation by distance (IBD = correlation between geographic and genetic distance; Wright 1943) or physical barriers causing a reduction of gene flow, leading to random changes in allele frequencies within a population, in the absence of natural selection. Selective pressures by environmental conditions, on the other hand, act on traits in a population, which can result in locally adapted populations (e.g. Burger and Lynch 1995; Kawecki and Ebert 2004). The process of local adaptation and the reduction of gene flow due to reduced fitness of dispersing individuals has been coined isolation by environment (IBE; Orsini et al. 2013). If selection pressures differ among populations, it will lead to adaptive genetic differentiation among populations enhanced by IBE. The balance between dispersal rate and the strength of selection dictates whether and how quickly populations become locally adapted. Even though local adaptation is possible in the presence of gene flow (Nosil and Crespi 2004), extensive evidence suggests that gene flow between environments posing divergent selection pressures on local populations can reduce local adaptation and instead drive adaptive phenotypic plasticity (theoretical: Hendry et al. 2001; Lenormand 2002; empirical: Nosil and Crespi 2004; Hemmer-Hansen et al. 2007; Räsänen and Hendry 2008).

One way to study the relative importance of IBD versus IBE is to assess the correlation between easily obtained neutral genetic markers, such as microsatellite markers, and both

environmental heterogeneity and geographic distance. The premise of this approach is that a reduction of gene flow will lead to genetic drift in neutral markers. Gene flow can be reduced by either geographic distance/physical barriers (IBD/isolation by distance, IBD) or by reduced fitness of dispersing individuals due to maladaptation to the new environment (IBE; Edelaar et al. 2012; Shafer and Wolf 2013; Wang et al. 2013; Sexton et al. 2014; Wang and Bradburd 2014). A classic example for IBE comes from plants growing on and near main tailings, where adaptation to different soil types has occurred (Antonovi 1968; and see also Antonovics 2006). Ever since Kimura (Kimura 1984) suggested 'the neutral theory of molecular evolution' (i.e. most evolutionary changes and most of the variation within and between species at the molecular level is not caused by natural selection but by random genetic drift) a big debate and a large pool of literature arose investigating population differentiation originating either by natural selection or by genetic drift. In this study, we investigate patterns of IBD, IBE and phenotypic population differentiation in a small marine fish, the common goby (*Pomatoschistus microps*) along an environmental gradient in the Baltic Sea. The lack of obvious barriers to gene flow in marine environments led to the general belief that the genetic population structure of marine organisms is homogenous (e.g. Ward et al. 1994). Over the past decade, however, several studies describe distinct genetic structuring for marine species on coarse and fine geographical scales (Pampoulie et al. 2004; Hoffman et al. 2005; Hauser and Carvalho 2008). Currents and climatic barriers, but also biotic and abiotic differences between locations, may restrict dispersal or result in local adaptations promoting genetic differentiation between populations.

The Baltic Sea provides an ideal study area to investigate recent and ongoing population divergent processes. The Baltic Sea originated only after the last glacial period (~ 10,000 years before present (BP)) at the end of the Pleistocene and passed since then through different

stages from brackish (Yolida Sea ~ 10,000 years BP) to fresh water (Ancyclus-Lake ~ 7,800 years BP) and again to a brackish stage (Littorina Sea ~ 5,000 years BP). Today it is characterised by a steep salinity gradient: decreasing in salinity from West (~ 20‰) to East (~ 3-4‰) and from South (~ 7-12‰) to North (~ 3-4‰; Stigebrandt 2001; Fig. 1). This natural gradient implies for marine species that only those, which could genetically adapt to low salinities or have a highly plastic response to salinity succeeded in colonising the Baltic Sea.

The common goby is an annual marine fish. The species is known to be highly adaptable to environmental fluctuations (Dolbeth et al. 2007) and can be found in salinities ranging from 0.5-35 ‰ in coastal waters and estuaries (Fonds and van Buurt 1974). Belonging to the Atlantic-Mediterranean sand goby group, its origin lies within the Mediterranean Sea where the oldest fossil remains have been found (Simonovic 1999; Tougard et al. 2014). There is evidence for a northward range expansion of common gobies ca. 10,000 years BP as well as for range contractions and expansions during earlier glaciations and interglacial periods (Gysels et al. 2004a).

A number of studies have dealt with the phylo-geographical patterns of species of the family Gobiidae, including marine species of the sand goby group to which *P. microps* belongs (Wallis and Beardmore 1984; Huyse et al. 2004; Gysels et al. 2004a, b; Larmuseau et al. 2009; Tougard et al. 2014). Gysels et al. (2004a) investigated colonisation patterns of *P. microps* using allozyme electrophoresis (CAGE) and mitochondrial cytochrome *b* haplotypes. In their study, however, they did not include any Baltic populations, so the phylogenetic structure of *P. microps* in the Baltic Sea is still unknown. Furthermore, it is suggested that recent and ongoing population divergent processes are more likely to be detected when using microsatellite markers instead of CAGE or mtDNA (Granevitze et al. 2014).

The body of literature is growing investigating the relative contribution of both neutral as well as adaptive evolutionary processes driving population divergence within a species (Hendry et al. 2002; Hendry and Taylor 2004; Nosil and Crespi 2004; Moore and Hendry 2005; Crispo et al. 2006; Minder and Widmer 2008; Brousseau et al. 2015; Ruiz-Gonzalez et al. 2015); far less studies exist doing so using a marine organism (Coscia et al. 2012; Nanninga et al. 2014; Giles et al. 2015; Guo et al. 2015; Tepolt and Palumbi 2015). We used eight variable microsatellite markers to investigate the population genetic structure of common gobies. Our main goal is it to assess the relative contribution of neutral versus adaptive evolutionary processes driving population divergence in the marine goby common goby with focus on Baltic populations. Specifically, we want to address (1) the genetic structure of common gobies in the Baltic Sea and compare it to three benchmark populations of the North, Atlantic and Mediterranean Seas, (2) the relative contribution of IBD versus IBE (salinity) on population divergence (using Mantel tests as well as Generalized Dissimilarity Models) and for Baltic populations (3) the roles of natural selection and genetic drift causing phenotypic population divergence by comparing phenotypic differentiation (P_{ST}) to genetic differentiation (F_{ST}). We expect to find that populations are genetically more differentiated from each other with increasing geographic distance (IBD), because common gobies dispersal is assumed only to occur during their larval stage (Jones and Miller 1966). Alternatively, if salinity poses strong selection, we expect to find IBE due to salinity. Furthermore, we expect to find divergence in phenotypic traits caused by local selection to different environments.

Methods

Study species

The common goby (*Pomatoschistus microps*; Fig. 2) is one of the most abundant fish species along the European coast (Healey 1972). The range of distribution of this small (TL 3-6 cm), benthic fish extends along the Atlantic coast from Morocco to central Norway including the Irish and British coasts as well as the North and Baltic Seas (Wheeler and Du Heaume 1969). The occurrence in the Mediterranean is limited to lagoons and estuaries in the Gulf of Lyon and Corsica (Bouchereau 1997a; Bouchereau and Guelorget 1998). Its dispersal is suggested to take place during the pelagic larval stage (Jones and Miller 1966) since adults are considered poor swimmers (Miller 1984). Gene flow between populations of the Mediterranean and different populations of the Atlantic appear to be restricted (Gysels et al. 2004a; Tougard et al. 2014). Common gobies are sexually dimorphic fish with a resource based mating system and paternal care (Kvarnemo et al. 1998).

Fish collection and sampling sites

Fish included in the genetic analyses were collected from eleven populations in the Baltic Sea in 2012 (population Poland (PU) collected in 2014; Table 3, Fig. 9). Adult fish were caught using a hand trawl (width 2 x 3m wings, funnel length 2.5 m, mesh size 4 mm in the wings and 2 mm in the funnel) in shallow waters near the coast. Fish were euthanized using an overdose of MS-222 and stored in 96% ethanol in a -80°C freezer. We also included fish from the North Sea (BR), southern Atlantic (MI) and Mediterranean (LV; see Table 3 for details) for genetic analyses. For the seven populations included in morphometric analyses, fish were collected in 2014 as explained above (see also Table 3 sites marked with *). Fish were photographed live

(see body shape analyses) after which all individuals were released back into their natural habitat.

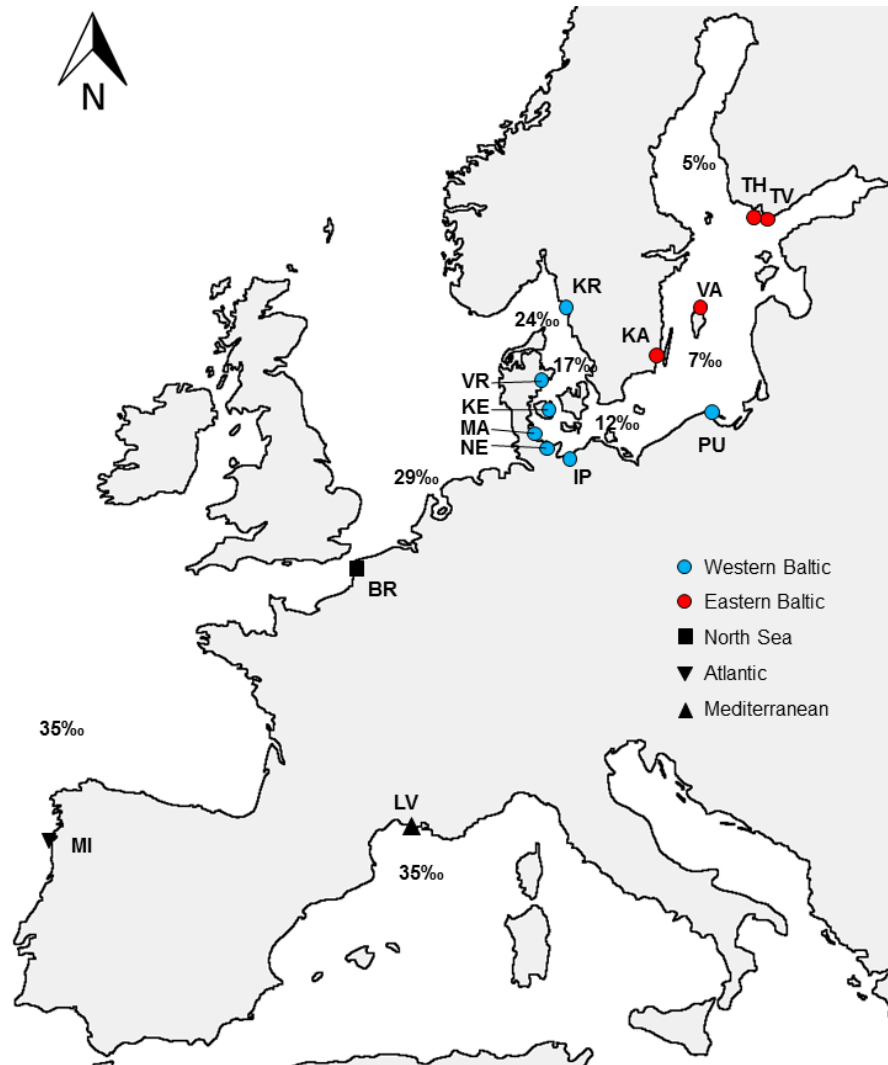


Figure 9. Fish collection sampling sites categorised based on Nei's genetic distance (Neighbour-Joining consensus tree). Included are western Baltic populations (●, 7), eastern Baltic populations (●, 4), North Sea (■, 1), Atlantic (▼, 1) and Mediterranean Sea (▲, 1) populations, salinity values (‰) and site abbreviations (see Table 3).

Table 3: Summary of sampled populations of *Pomatoschistus microps* included in this study. Reported are: sampling sites, abbreviations for sites (Abb.), sample size (N), groups (WBaltic: western Baltic, EBaltic: eastern Baltic), geographic locations, mean salinity (in ‰), coordinates (Latitude, Longitude), dates of sampling (month-year). Asterisks denote populations included in the geometric morphometric analyses of body shape.

Sampling site	Abb.	N	Group	Geographic location	Salinity (PSU)	Latitude	Longitude	Dates
Kristineberg *	KR	20	W Baltic	western Sweden, Gullmarfjord	22.2	58°24'N	11°46'E	May-12
Vrinnars	VR	10	W Baltic	Denmark, Bay of Arhus	20.3	56°15'N	10°30'E	Jul-12
Kerteminde*	KE	19	W Baltic	Denmark, Fuenen	16.8	55°44'N	10°65'E	Jul-12
Maasholm	MA	23	W Baltic	Germany, Schlei estuary	14.2	54°69'N	9°99'E	Jun-12
Neustadt	NE	23	W Baltic	Germany, Bay of Mecklenburg	13.3	54°69'N	10°80'E	Sep-13
Island Poel*	PO	24	W Baltic	Germany, Bay of Mecklenburg	12.2	53°99'N	11.48'E	Jun-12
Puck*	PU	23	W Baltic	Poland, Puck Bay	7.3	54°43'N	18°24'E	May-14
Kalmar*	KA	24	E Baltic	eastern Sweden, Kalmar strait	7.4	56°66'N	16°36'E	Jun-13
Valleviken*	VA	22	E Baltic	Island Gotland, Sweden	7.2	57°78'N	18°94'E	Jun-12
Tvärminne (Henriksberg)*	TH	21	E Baltic	south-western Finland, Gulf of Finland	5.8	59°82'N	23°14'E	Jun-12
Tvärminne (Vindskar)	TV	17	E Baltic	south-western Finland, Gulf of Finland	5.8	59°82'N	23°21'E	Jun-12
Branst	BR	24	North Sea	Belgium, Schelde estuary	29	51°61'N	4°10'E	Oct-14
Minho	MI	15	Atlantic	northern Portugal, Minho estuary	35	41°52'N	8.51'E	na
Lagoon Vaccarés	LV	22	Mediterranean	southern France, Rhone estuary	35	43°25'N	4°55'E	na

Genetic analyses

Total genomic DNA was extracted from caudal fin clips of all 287 individuals in each of the 14 sampled locations using a DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). Nine microsatellite markers previously used to characterize members of the sand goby group were individually labelled at the 5' end with fluorescent dye and multiplexed on 3 separate panels (see Table 4 for details). Locus *Pmin09* was run independently due to low signal strength when multiplexed. Microsatellite markers were amplified using polymerase chain reaction (PCR, 10 µl final volume) carried out in an Eppendorf Mastercycler® thermocycler (Eppendorf AG, Hamburg, Germany). PCR conditions for each reaction using the Qiagen® Multiplex PCR kit were 5 µl of Qiagen Master Mix (containing HotStarTaq DNA polymerase, dNTPs and 3mM MgCl₂), 0.1 µM of each primer (forward and reverse), 2.8 µl HPLC water, and 2 µl extracted template DNA. The thermal cycling profile consisted of an initial denaturation for 5 min at 95°C followed by 30 sec of 95 °C, 60 °C reannealing temperature (90 sec), 30 cycles at 95°C, an extension step at 72 °C (30 sec) and a final extension at 60°C for 30 min. Fragment lengths of PCR products were analysed using an Applied Biosystems® (ABI, Life Technologies GmbH, Darmstadt, Germany) 3730 capillary sequencer and visualized using ABI GENEMAPPER v. 5.0 software.

Microsatellite data were analysed with GENEPOP 4.2 (Raymond and Rousset 1995) to test for Hardy-Weinberg (HW) equilibrium (Fisher's exact test) and for genotypic linkage disequilibrium for pairs of loci within populations (Fisher's exact test). Sequential Bonferroni corrections (Rice 1989) were carried out for both tests mentioned above. Number of alleles (N_a) as well as observed and expected heterozygosity (H_o and H_e) were calculated using GenAlEx 6.501 (Peakall and Smouse 2006; Peakall and Smouse 2012). Allelic richness (A_r) was

calculated using FSTAT (Goudet 1995). We tested microsatellite loci for neutrality with the software LOSITAN (Antao et al. 2008) within each population. To visualize genetic relationships between populations, a Neighbour-Joining (NJ) tree was assembled based on Nei's genetic distance (D_A ; Nei 1972) with 10,000 bootstrap replicates for each loci, drawn with the program POPTREE2 (Takezaki et al. 2010) and edited in MEGA 6.0 to create a 50% majority tree (Tamura et al. 2013).

Table 4: Microsatellite information. Locus name, fluorescent labels at the 5' end, panel name, number of different alleles per locus (N_a), species and the reference of the first description for each microsatellite locus.

Locus	5' labels	Panel	N_a	Species	Reference
<i>Pmic02</i>	VIC	2	53	<i>P. microps</i>	Berrebi et al. 2006
<i>Pmic03</i>	NED	2	15	<i>P. microps</i>	Berrebi et al. 2006
<i>Pmin04</i>	NED	3	6	<i>P. minutus</i>	Larmuseau et al. 2007
<i>Pmin05</i>	6fam	3	20	<i>P. minutus</i>	Jones et al. 2001
<i>Pmin09</i>	VIC	na	38	<i>P. minutus</i>	Berrebi et al. 2006
<i>Pmin35</i>	NED	1	20	<i>P. minutus</i>	Larmuseau et al. 2007
<i>Pmin38</i>	6fam	1	8	<i>P. minutus</i>	Larmuseau et al. 2007
<i>Pmar03</i>	6fam	3	39	<i>P. marmoratus</i>	Berrebi et al. 2006
<i>Pmar05</i>	6fam	2	32	<i>P. marmoratus</i>	Berrebi et al. 2007

To determine the population genetic structure using Bayesian clustering, the program STRUCTURE 2.3.4. (Pritchard et al. 2000) was implemented using the admixture model. STRUCTURE determines the most likely number of populations (K) with gene frequencies in HW and linkage equilibrium, using a Bayesian, model-based algorithm. Each run consisted of a burn-in period of 200,000 and 500,000 Markov Chain Monte Carlo (MCMC) repetitions. Possible numbers of clusters (K) ranged from $K = 1$ to $K = 14$ which equals the maximum

number of sites sampled. Each value of K had ten separate iterations. The software STRUCTURE HARVESTER A.2 (Earl and vonHoldt 2012) was used to determine the most likely value of K (at which ΔK is maximal; Evanno et al. 2005). To find the optimal alignment of the results from the 14 replicate cluster analyses, we used the software package CLUMPP 1.1.2b (Jakobsson and Rosenberg 2007). The results from STRUCTURE and CLUMPP analyses were summarized using the program DISTRUCT (Rosenberg 2004).

Genetic differentiation, measured by F_{ST} , was calculated between each population pair with ARLEQUIN 3.5.1.2 (Excoffier and Lischer 2010). Significance of global pairwise F_{ST} was estimated with ARLEQUIN using 10,000 permutations. Wright's F statistic (1943) decreases with increasing allelic diversity (Wright 1951; Hedrick 2005; Jost 2008; Meirmans and Hedrick 2011); therefore we directly compare the fixation index, F_{ST} , and an index of genetic differentiation, D_{est} (Jost 2008). The pure index of genetic differentiation was calculated with SMOGD 1.2.5. (Crawford 2010). To test for genetic isolation by distance (IBD), pairwise F_{ST} and D_{est} estimates calculated between samples were plotted against geographical distance using a paired Mantel test (Mantel 1967) implemented in GenAlEx 6.501. The significance of observed associations was evaluated using 1,000 permutations. The Mantel test uses a non-spatial Island model (Wright 1931), which assumes equal migration among all populations regardless of their distance as well as independence of samples. To explicitly evaluate the importance of geographic distance between populations (IBD) and the influence of salinity (IBE) on population divergence in *P. microps*, we performed additionally a Generalized Dissimilarity Model (GDM) in R (Ferrier et al. 2007). GDMs take into account the influence of geographic distance on explaining biological variation. We ran GDMs including the response variable (F_{ST} or P_{ST}) to evaluate genetic dissimilarities between populations and two predictor variables: (1) shortest geographic distance between populations through water (overwater distance) and

(2) salinity as environmental variable. Additional fitted functions of population groupings following the hierarchical approach based on the NJ tree are reported: (1) all populations, (2) Atlantic, North Sea, Baltic Sea, (3) North Sea, Baltic Sea and (4) Baltic Sea.

P_{ST} - F_{ST} comparison

The degree of genetic population differentiation can be measured with neutral molecular markers using Wright's fixation index, F_{ST} (Wright 1943). Low F_{ST} values indicate high gene flow between populations possibly hindering local adaptation. High F_{ST} values, on the other hand, can be caused by genetic drift due to limited gene flow.

For a neutral quantitative trait that has an additive genetic basis, the analogue to F_{ST} is Q_{ST} (Spitze 1993):

$$Q_{ST} = \frac{V_{A,b}}{V_{A,b} + 2V_{A,w}},$$

where $V_{A,b}$ and $V_{A,w}$ are the morphological additive genetic components between and within populations.

Q_{ST} - F_{ST} comparisons are commonly used to distinguish between natural selection and genetic drift as causes of population differentiation (Leinonen et al. 2013). For a quantitative trait under divergent selection $Q_{ST} > F_{ST}$ and different phenotypes are favoured, under convergent selection $Q_{ST} < F_{ST}$ and the same phenotypes are favoured in different populations. Difficulties in estimating within- and among-population components of genetic differentiation in quantitative traits made it common to replace Q_{ST} with its phenotypic analogue P_{ST} (Leinonen et al. 2006; Brommer 2011; Leinonen et al. 2013):

$$P_{ST} = \frac{V_b}{V_b + 2V_w}$$

Two parameters, c (proportion of the total variance that is presumed to be due to the additive genetic effects across populations) and h^2 (heritability, the proportion of phenotypic variance due to additive genetic effects), determine the accuracy of the approximation of Q_{ST} by P_{ST} .

$$\frac{c}{h^2} = \frac{F_{ST}(P_{ST} - 1)}{P_{ST}(F_{ST} - 1)}$$

We followed Whitlock and Guillaume (2009) when comparing phenotypic differentiation (P_{ST}) to neutral molecular differentiation (F_{ST}). We adapted the R script provided as appendix in Whitlock and Guillaume (2009) for the use of P_{ST} instead of Q_{ST} and ran 1,000 iterations to obtain confidence intervals for F_{ST} and P_{ST} estimates. As P_{ST} depends on the ratio between (c) among and within (h^2) population additive genetic variation, we calculated P_{ST} (and its confidence interval) for values ranging from 0.05 to 1. Canonical variate scores of body shape analyses of males and females (see below) were used to calculate the within (V_w) and the between (V_b) phenotypic variance using an ANOVA.

Geometric morphometrics were carried out on 134 female and 141 male photographs of common gobies of seven Baltic populations (see Table 3 sites marked with *). Photographs were taken with a Canon EOS 1100D digital SLR camera of live fish resting in a water filled photo chamber (5cm x 5cm x 1.5cm) made of black polyethylene with a glass front. Fish were photographed on the left side, and with a moveable glass plate gently pushed towards the glass front of the chamber to make sure they are aligned parallel. To analyse the body shape of each fish, we chose 15 landmarks (lm) following general guidelines for placement of lms (Klingenberg and McIntyre 1998; Fig. 10). Lm's were digitized using the software TpsDig (Rohlf 2006). To indicate how bent or straight each fish was we calculated five angles: the angle of lm 4 and lm 9 as well as the angles at the midpoints between lm 5 and lm 14, lm 6 and lm 13, and between lm 7 and lm 12 (personal communication C. Klingenberg). These angles were

used as covariates in a regression on body shape coordinates after a Procrustes superimposition using the software MorphoJ (Klingenberg 2011). We created subsamples ($N = 7$) of each population separated by sex. To account for any level of allometric size variation caused by size differences of fish from different populations, we corrected the residuals of body size to the log centroid size. The residuals corrected for allometric size differences were then used in a canonical variate analyses (CVA; Albrecht 1980), which produces new variables, the canonical variates (CVs). To see the main patterns among groups it is often sufficient to display two or three CVs. Here, only CVs with an Eigenvalue greater than one are included (see Kaiser 1960).

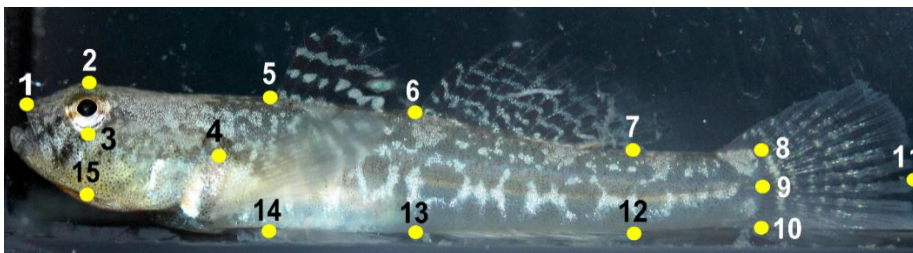


Figure 10. Landmark (lm) configuration (lm 1-15) on a male common goby digitised using the software tpsDig (Rohlf, 2006) to analyse body shape.

Results

Genetic analyses

The mean number of alleles per locus was 48.3 for western Baltic and only 18.9 for eastern Baltic populations (see S1 also for results on Mediterranean, Atlantic, North Sea populations).

The observed heterozygosity (H_o) showed slightly lower average values for western and slightly higher values for eastern Baltic populations (see S1). Average allelic richness (A_r) was found to be higher among western Baltic (36.5) than among eastern Baltic (14.3) populations

(see S1). Only one case involving deviation from HW equilibrium was observed, which could not be accounted for after Bonferroni correction (Pop: NE, locus: *Pmar05*). Significant linkage disequilibrium was found between *Pmic02* and *Pmar05*, and *Pmic02* and *Pmin09*. After removing *Pmic02* there was no linkage disequilibrium observed after Bonferroni correction for multiple tests. *Pmic02* was excluded from all further genetic analyses. Analyses of loci over all populations for neutrality with LOSITAN (Beaumont and Nichols 1996) suggest that loci are neutral, except locus *Pmin38*, which shows a signature of positive divergent selection. *Pmin38* was therefore excluded from analyses of the P_{ST} - F_{ST} comparison.

Genetic differentiation between population pairs, estimated as pairwise F_{ST} and D_{est} , ranged from - 0.003 to 0.365 for F_{ST} and - 0.001 to 0.528 for D_{est} (Table 5). The highest F_{ST}/D_{est} values are found for populations pairing with the Mediterranean population (LV). For eastern Baltic populations, F_{ST} ranged from 0.002 to 0.061 and D_{est} from - 0.001 to 0.047. For western Baltic populations F_{ST} ranged from - 0.003 to 0.025 for F_{ST} and D_{est} from - 0.001 to 0.026. The average genetic differentiation among eastern Baltic populations ($F_{ST} = 0.036 / D_{est} = 0.025$) is higher than among western Baltic populations ($F_{ST} = 0.003 / D_{est} = 0.002$). The genetic differentiation between populations within the eastern and the western Baltic clade is generally very small (see also e.g. Pocwierz-Kotus et al. 2015), however, among eastern Baltic populations it is about an order of magnitude larger than that seen among western Baltic populations.

Chapter II

Table 5. Population pairwise global F_{ST} and pure genetic differentiation index D_{est} (F_{ST}/D_{est} below the diagonal). F_{ST} values tested by 1023 permutations. **Non**-significant pairwise differences ($\alpha = 0.05$) are marked with an asterisk. Overwater distance (minimum distance between sites through water connection in km) between sample sites above the diagonal. See Table 3 for site abbreviations.

Region		KR	KE	VR	MA	NE	IP	PU
W Baltic	KR		318.4	247.9	420.8	456.6	463.0	760.5
W Baltic	KE	-0.004/-0.001		86.9	110.2	146.7	183.0	747.4
W Baltic	VR	-0.003/0.000	-0.005/0.000		174.4	233.2	267.6	776.7
W Baltic	MA	0.000/0.004	0.000/0.001	-0.015/-0.026		110.6	182.0	659.8
W Baltic	NE	0.006/0.002	-0.004/0.000	-0.011/-0.009	0.003/0.000		75.8	646.8
W Baltic	IP	0.000/-0.001	0.002/0.000	-0.003/0.000	0.004/0.003	0.002/0.000		605.0
W Baltic	PU	0.009/0.010	0.015/0.011	0.015/0.006	0.025/0.026	0.009/0.015	0.004/0.000	
E Baltic	KA	0.128/0.056	0.108/0.051	0.136/0.052	0.128/0.087	0.113/0.060	0.091/0.052	0.093/0.050
E Baltic	VA	0.086/0.042	0.055/0.043	0.077/0.033	0.077/0.052	0.060/0.025	0.062/0.055	0.086/0.087
E Baltic	TH	0.104/0.115	0.094/0.086	0.108/0.065	0.095/0.050	0.088/0.078	0.077/0.079	0.082/0.111
E Baltic	TV	0.080/0.068	0.068/0.056	0.072/0.037	0.061/0.023	0.068/0.054	0.043/0.036	0.056/0.064
North Sea	BR	0.025/0.046	0.028/0.038	0.020/0.040	0.018/0.042	0.034/0.072	0.022/0.050	0.040/0.077
Atlantic	MI	0.018/0.010	0.023/0.020	0.021/0.024	0.024/0.038	0.026/0.041	0.018/0.019	0.033/0.015
Mediterranean	LV	0.177/0.302	0.193/0.313	0.203/0.354	0.189/0.319	0.192/0.368	0.201/0.356	0.213/0.411

Chapter II

Region		KA	VA	TH	TV	BR	MI	LV
W Baltic	KR	571.6	834.1	1309.9	1317.2	978.8	2912.8	4886.7
W Baltic	KE	649.2	956.2	1464.8	1471.8	1222.5	2653.8	4936.4
W Baltic	VR	659.1	959.1	1461.8	1468.9	1299.7	2687.0	4996.8
W Baltic	MA	740.5	1049.9	1560.8	1567.7	1432.2	2546.3	5165.8
W Baltic	NE	677.4	987.6	1499.9	1506.6	1472.0	2585.4	5197.9
W Baltic	IP	615.5	924.7	1436.7	1443.3	1509.0	2641.6	5252.5
W Baltic	PU	390.5	467.8	798.3	803.3	2034.4	3711.2	5777.0
E Baltic	KA		310.4	822.6	829.3	2061.2	3255.7	5668.6
E Baltic	VA	0.045/0.027		512.3	519.0	2234.3	3562.1	5940.0
E Baltic	TH	0.033/0.025	0.060/0.047		7.4	2556.1	4070.7	6287.3
E Baltic	TV	0.020/0.017	0.052/0.037	0.001/-0.001		2560.1	4076.9	6292.0
North Sea	BR	0.144/0.194	0.098/0.175	0.112/0.150	0.072/0.111		1733.9	3870.4
Atlantic	MI	0.140/0.148	0.078/0.067	0.127/0.221	0.085/0.113	0.024/0.015		2303.9
Mediterranean	LV	0.365/0.528	0.279/0.377	0.330/0.554	0.293/0.471	0.172/0.362	0.126/0.214	

The neighbour-joining (NJ) tree based on Nei's genetic distance (D_A ; Fig. 11) confirms the existence of four genetically differentiated clusters: the (1) Mediterranean and the (2) Atlantic cluster (bootstrap support: 100%), the (3) North Sea (bootstrap support: 98%) and the (4) Baltic Sea cluster (bootstrap support: 86%). Interestingly, low-salinity, eastern Baltic populations form a monophyletic group (bootstrap support: 94%) within the Baltic cluster. Clusters visualised in the NJ-tree agree with the geographical origin of the particular populations (Table 3).

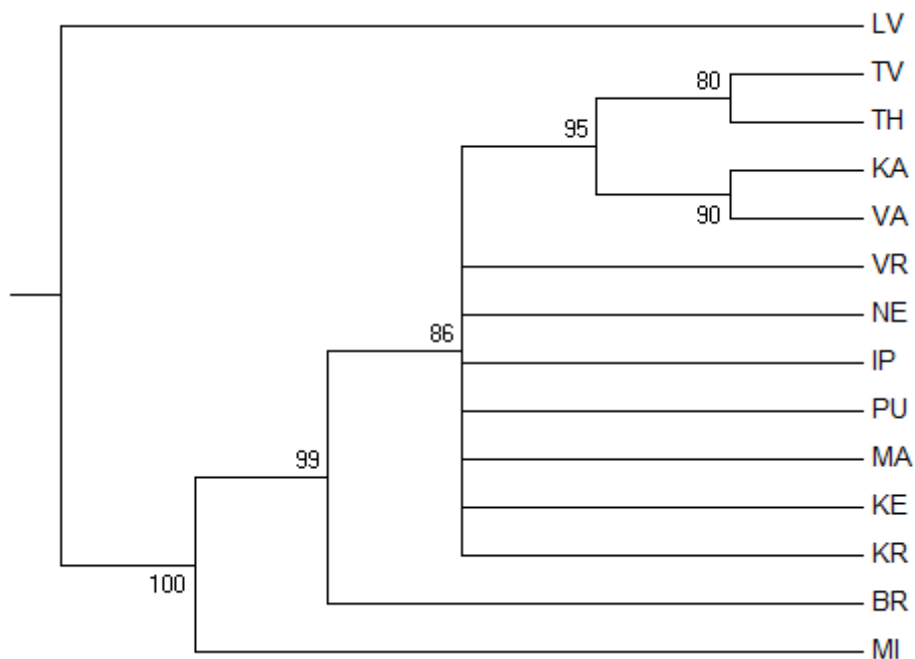


Figure 11. Neighbour Joining (NJ) consensus tree with percentage of bootstrap support > 50% of 10,000 replicates showing the genetic relationships between populations based on Nei's genetic distance (D_A) across all eight microsatellite loci. The tree was edited to collapse branches with bootstrap support < 50%. See Table 3 for site abbreviations.

The results of the STRUCTURE analyses provide evidence for three genetic clusters ($K = 3$; Fig. 12) corresponding to the (1) Mediterranean Sea, the (2) Atlantic/North Sea/western Baltic and

the (3) eastern Baltic populations. High gene flow between the Atlantic/North Sea and western Baltic populations as well as high gene flow between eastern and western Baltic populations was suggested, but only limited gene flow between the Atlantic/North Sea and the eastern Baltic clusters. STRUCTURE was run for all 14 sites with and without the Mediterranean population ($K = 1-13$) and separately for the Atlantic, North Sea, western Baltic ranges ($K = 1-9$), the North Sea and Baltic Sea ($K = 1 - 12$), as well as for the Baltic populations alone ($K = 1-11$) and for the eastern Baltic ($K = 1-4$) and western Baltic ($K = 1-7$) populations separately. However, additional STRUCTURE hierarchical analyses revealed no further clustering within the major regions (results not shown).

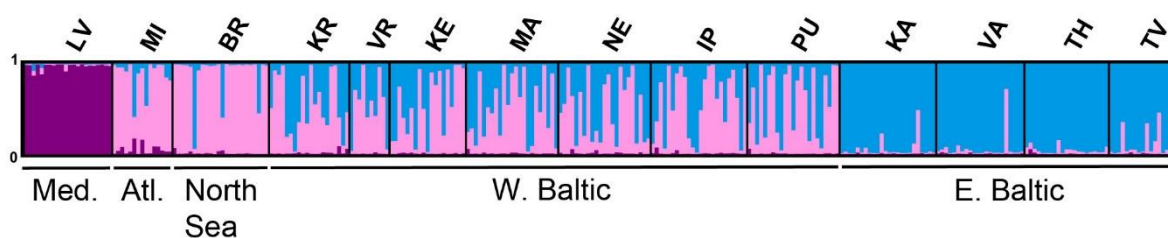


Figure 12. Estimated genetic structure of all 14 populations included in the analysis, $K = 3$. STRUCTURE bar plots (aligned using DISTRUCT) where each individual is represented by a vertical line and membership of each individual to the different clusters can be deduced from the proportion of each colour in the bars. Populations are labelled above the figure. See Table 3 for site abbreviations.

There was a significant positive association between geographic distance and genetic distance (F_{ST}/D_{est}) shown by the Mantel test (F_{ST} : $R^2 = 0.690$, $P < 0.0001$; D_{est} : $R^2 = 0.794$, $P < 0.0001$; see also Table 6). The positive IBD pattern was strongly influenced by the major regions (i.e. Mediterranean Sea, Atlantic Ocean, North Sea, and Baltic Sea). Results of the Mantel test for different groupings of regions showed a positive correlation between geographic and genetic distance except for the western and eastern Baltic clade (see Table 6).

Table 6. Paired Mantel test results for correlation between genetic (F_{ST} and D_{est}) and geographic distances for different groupings of populations (group). P-values derive from 10,000 permutations. Significant results ($\alpha = 0.05$) are given in bold. See Table 3 for details about groups.

Group	F_{ST}		D_{est}	
	R^2 - values	P-value	R^2 - values	P-value
All 14 populations	0.690	< 0.0001	0.794	< 0.0001
Atlantic/North Sea/Baltic Sea	0.128	0.050	0.338	< 0.0001
North Sea/ Baltic Sea	0.275	0.003	0.572	0.001
Baltic populations	0.382	0.006	0.467	0.004
W. Baltic populations	0.352	0.064	0.396	0.056
E. Baltic populations	0.367	0.161	0.255	0.152

To evaluate the importance of IBD but at the same time incorporating salinity as environmental variable (IBE) we performed GDMs (Fig. 13) for different groupings of major regions (based on F_{ST} , and for Baltic populations also on P_{ST}). Including all populations ($N = 14$; Fig. 13a) in the model, IBD explained genetic dissimilarity between populations best (67.4%). However, running the model without the Mediterranean population (Fig. 13b) we found that IBE using the selective agent 'salinity' explains the genetic divergence between Atlantic/North Sea/Baltic Sea (48.6%) best. When testing genetic dissimilarity between the sister groups, North Sea and Baltic Sea (Fig. 13c), we again found salinity to explain a large portion of the genetic divergence between these clusters (47.4%). Within the Baltic Sea (results not shown) however using F_{ST}/P_{ST} as response variable, neither IBD nor IBE could explain genetic differentiation between populations.

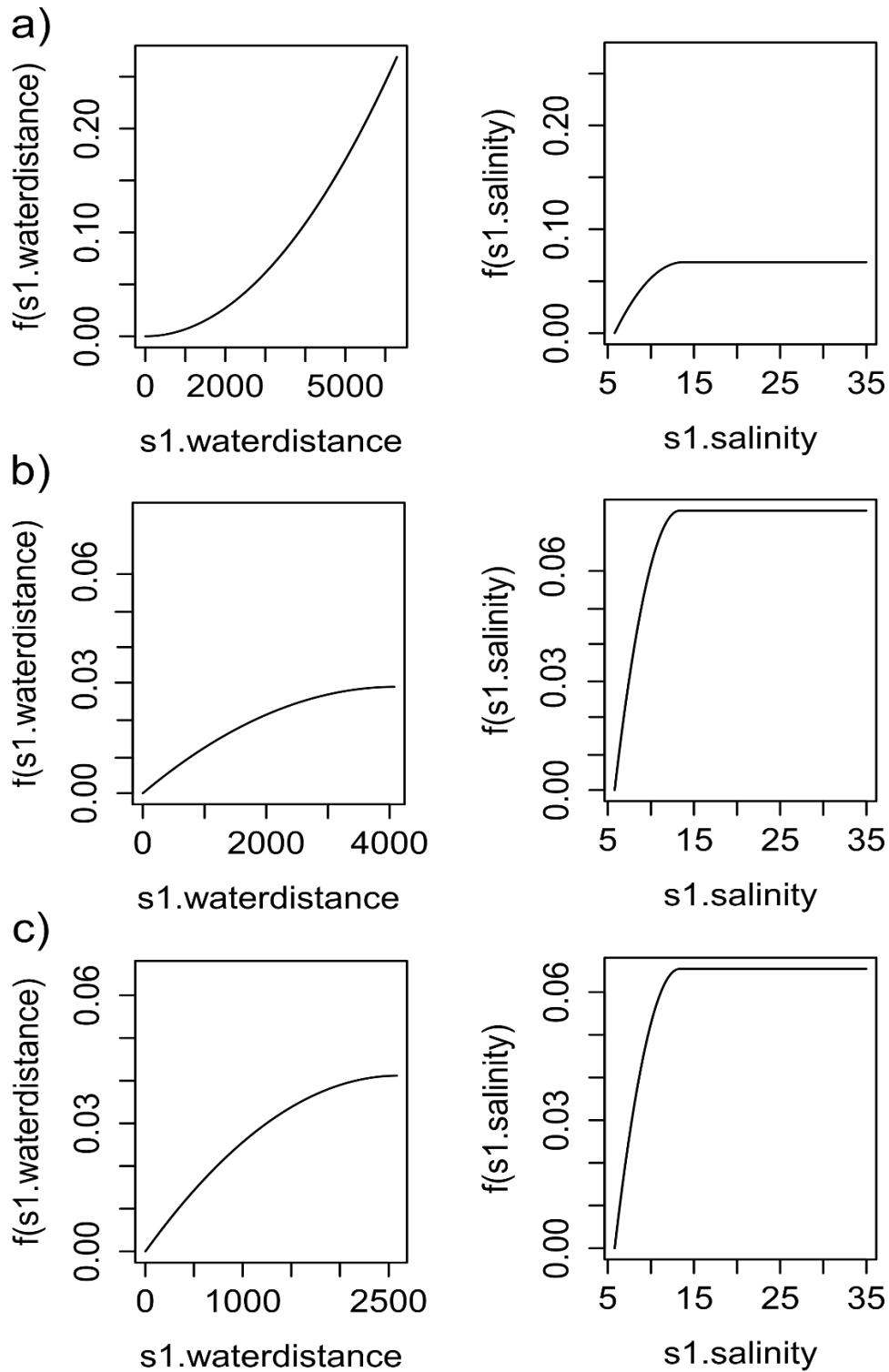


Figure 13. Fitted functions for the two predictor variables ‘water distance’ (left) and ‘salinity’ (right) for groupings of populations, following the suggested genetic structure of the Neighbour-Joining consensus tree based on Nei’s D_A (see Fig. 11) for a) all populations, b) Atlantic/North Sea/Baltic Sea populations and c) North Sea/Baltic Sea populations.

P_{ST}- F_{ST} comparison

Genetic differentiation between Baltic populations for both sexes was very low (Table 5). For almost all c/h^2 ratios, P_{ST} was greater than F_{ST} for both sexes and all traits (Fig. 14 a-c). More importantly, 95% confidence intervals for P_{ST} and F_{ST} did not overlap for most values of c , indicating that P_{ST} values were credibly larger than F_{ST} values. We also tested if there was a relation between geographic distance and phenotypic differentiation of Baltic common gobies for males and females separately using a Mantel test (Table 6). Neither for males (P_{ST}: $R^2 = 0.006$, $P = 0.414$) nor for females (P_{ST}: $R^2 = 0.018$, $P = 0.285$) was a positive association found between phenotypic differentiation of body shape and geographic distance.

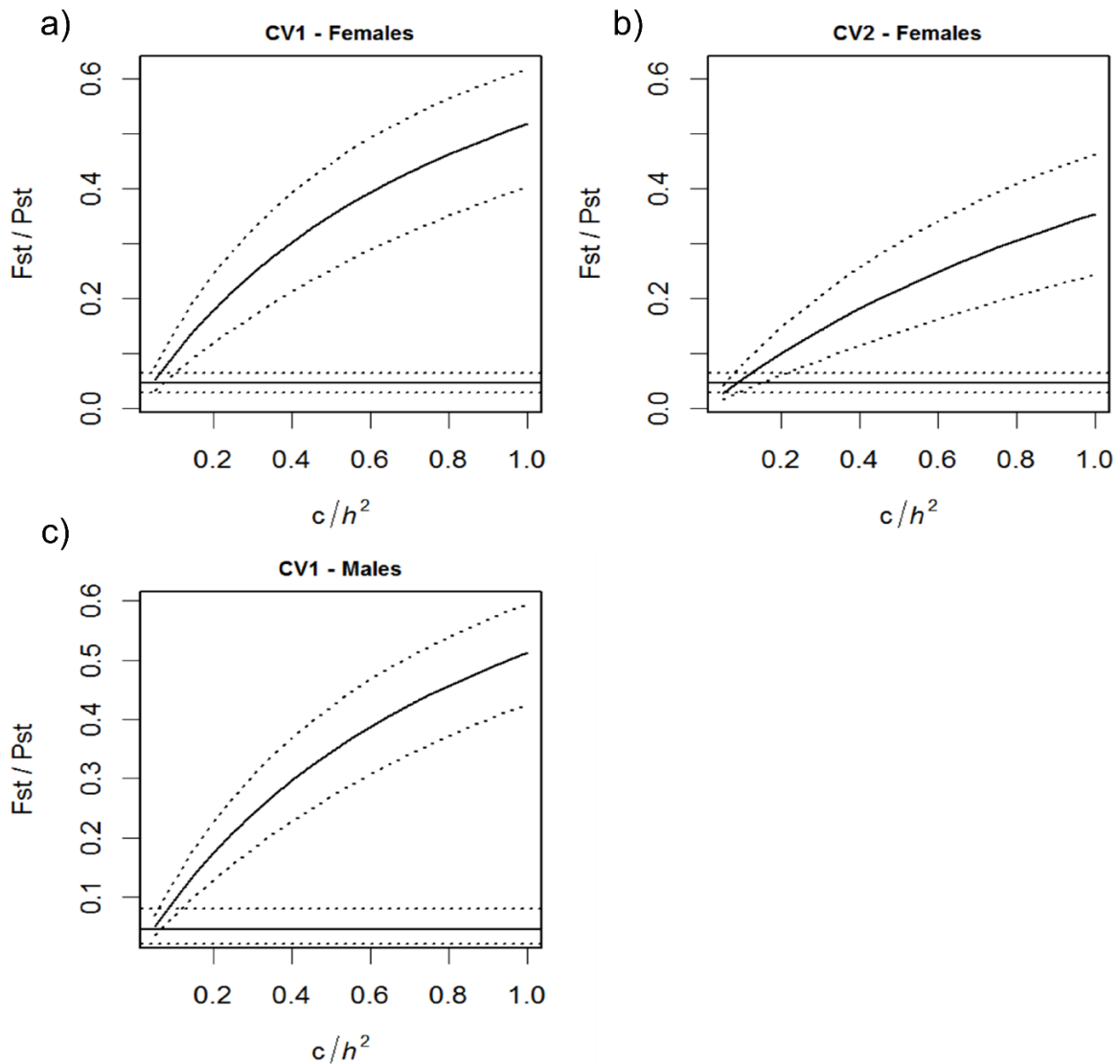


Figure 14. The relationship between observed phenotypic (P_{ST} - solid lines: slope) and neutral genetic differentiation (F_{ST} - solid lines: vertical) both with their upper and lower 95% confidence intervals (dotted lines) as function of body shape to among-population differentiation (c) and within population differentiation (h^2) of seven Baltic common goby populations for a) CV1 (females), b) CV2 (females) and c) CV1 (males). See Table 3 for details on populations used for geometric morphometric analyses on body shape.

Discussion

We here investigated population structure and drivers of divergence in common gobies, and our main findings are: First, common gobies show a clear genetic structure on both coarse (Mediterranean, Atlantic and North Sea) and fine (Baltic Sea) geographic scales. Second, the major drivers of this genetic structure are scale dependent, with IBD most important at coarse scales, and IBE (salinity) at finer scales. Finally, phenotypic population divergence in Baltic common gobies is driven by natural selection rather than genetic drift alone. In the following we will discuss each finding in turn.

Genetic structure of common gobies on coarse and fine spatial scales

Genetic clustering analyses using STRUCTURE revealed that three distinct genetic clusters are present (i.e., Mediterranean, Atlantic/North Sea/western Baltic, and eastern Baltic Sea), with high levels of admixture between the western Baltic and eastern Baltic populations as well as between western Baltic and Atlantic/North Sea. The admixture within the Baltic was also reflected in the NJ tree, where eastern Baltic populations form a monophyletic clade, however its position with respect to western Baltic populations' remains unclear (i.e., unresolved nodes within the Baltic). With these results we add to a slowly and just recently growing pool of literature showing that Baltic organisms can show a genetic differentiation between western (high salinity) and eastern (low salinity) Baltic origin (Holmborn et al. 2011; Olsson et al. 2012).

In addition, the phylogenetic tree suggested the Atlantic and North Sea populations to be distinct from one another and from the (western) Baltic populations. These slightly different results could be explained by the differences in the approaches; while STRUCTURE assigns individuals to clusters based on individual probabilities and admixture, a NJ-tree estimates genetic distances between populations. High genetic similarity of Atlantic and North Sea populations (STRUCTURE results) suggest high levels of gene flow between these populations. Nevertheless, genetic differences (F_{ST}) between Atlantic and North Sea populations (NJ-tree) contain sufficient information to suggest that they form distinct genetic clusters, similar to findings of Gysels et al. (2004a). However, their results are not directly comparable to ours, since their use of mtDNA and allozyme markers may not be sufficient to detect recent or ongoing population divergence. The complete genetic separation of the Mediterranean cluster suggested by clustering results as well as by the NJ-tree has already been shown previously (Gysels et al. 2004a) and might be a result of IBD (see below) but more likely is caused by the Almeria-Oran Oceanic Front (AOOF), the frontal zone where Atlantic and Mediterranean water bodies meet, which for many species poses a barrier for Atlantic-Mediterranean dispersal (Naciri et al. 1999). Since dispersal of *P. microps* is assumed to happen during the larval stage (Bouchereau 1997b), strong currents at the AOOF may therefore prevent Atlantic-Mediterranean dispersal and lead to the genetic isolation of Mediterranean populations.

When following the assumed route of post-last glacial maximum population expansion of *P. microps* (see Gysels et al. 2004a; Tougard et al. 2014) from the Mediterranean Sea into the Baltic Sea, F_{ST} values are highest in the Mediterranean but show a general decrease northwards. Lowest F_{ST} values are found between western Baltic populations, while F_{ST} is increasing at the step from the western Baltic to eastern Baltic populations, mirroring the

decrease in gene flow between western and eastern Baltic Sea. All western Baltic populations show a higher pairwise F_{ST} with Atlantic and North Sea populations than they do with any of the eastern Baltic populations. Allelic richness also decreases along the northward dispersal route of *P. microps*. However, in the Mediterranean allelic richness is as low as in eastern Baltic populations potentially due to the restricted geographic distribution. We assume that the most eastern Baltic populations of *P. microps* have colonized the Baltic Sea most recently. The founder population, in this case eastern Baltic populations, normally does not carry all alleles of the original population and therefore often shows lower allelic richness than the original population (Nei et al. 1975; Templeton 1980; Dlugosch and Parker 2008). Our results might show such a ‘founder effect’ among eastern Baltic populations originating from western Baltic populations, with a lower allelic richness among eastern Baltic than among all other populations (besides the Mediterranean population; Table S1). Due to drift, caused or enhanced by IBE, some alleles common in the original population may be completely absent in the new populations, while others might reach high frequencies. Supporting this explanation is the fact that low salinity eastern Baltic populations form a monophyletic group within the Baltic cluster. Overall, we found a genetic pattern that is gradual in space supporting the assumption of a northward dispersal route of common gobies.

Neutral versus adaptive evolutionary processes driving population divergence

Population divergence shown by differences in F_{ST} between populations can be explained by IBD and/or IBE. Paired Mantel tests suggested that IBD is the most important driver for population divergence for different constellations of major regions. Within Baltic populations, however, IBD does not explain genetic differentiation. This result might be due to the relatively low geographic distance between the tested populations and potentially restricted

gene flow to other populations. Recently, the Mantel test as well as the partial Mantel test (results not included here) has come under scrutiny (Meirmans 2012), because spatial dependency in the data is not taken into account as the null model is based on a non-spatial island model (Mantel 1967). Therefore, a Mantel test alone seems not to be a suitable method to evaluate the influence of IBD on population divergence in Baltic *P. microps*.

We also investigated salinity as a driver for IBE since salinity varies greatly over the distribution of *P. microps* is affecting several other ecological factors e.g. abundance and size of mussel shells required as nesting resource (see Chapter I; Forsgren et al. 1996b). We, therefore, also performed GDMs to take into account geographic distance between populations as well as salinity to assess the relative contribution of IBD and IBE causing population divergence. Geographic distance (IBD) was suggested to be the most important driver of population divergence when all populations were included. Salinity (a likely driver of IBE) however, was found to be best correlated to population differentiation between the Atlantic, North Sea and Baltic populations as well as the genetic differentiation of the sister groups, North Sea and Baltic Sea. Results clearly showed that besides IBD, IBE also plays an important role in driving population divergence. Comparing GDM results with the paired Mantel test results, it becomes clear that the Mantel test alone is often not reliable since it does not take into account any ecological variables possibly affecting spatial structuring of populations. We want to emphasise that GDM results represent a more reliable explanation to what extent IBD versus IBE are affecting population divergence of common gobies along their original dispersal route from the Mediterranean into the Baltic Sea. We expected within the Baltic Sea that IBE rather than IBD would be the main driver of population divergence due to the steep salinity gradient from West to East. GDM results however, reveal that within the Baltic Sea neither IBD nor IBE can explain population divergence. One possible explanation for that could be an

ecological 'isolation by colonisation' (IBC) scenario (Orsini et al. 2013), which is neutral and therefore no prediction can be made for non-neutral genetic variation neither in relation to space nor environment.

A selective agent such as salinity can reduce gene flow and as a result increase divergence between populations due to maladaptation of dispersing individuals to low respectively high salinity (see e.g. Fuller et al. 2007; Orsini et al. 2013). Variable water inflow from the North Sea into the Baltic Sea creates transition zones with fluctuating salinity (Skagerrak and Kattegat) which implies that selection for either high or low salinity will be absent and therefore no local adaptation to high/low salinities and correspondingly genetic population structuring is expected. However, in the eastern Baltic Sea salinity is constantly very low, possibly promoting local adaptation due to strong selection on low salinity reducing gene flow. Limited gene flow under high selection pressure is generally expected to facilitate local adaptation to different environments (theoretical: e.g. Hendry et al. 2001; Räsänen and Hendry 2008; empirical: e.g. Nosil 2009) and eastern Baltic populations forming a monophyletic group within the Baltic cluster might be the outcome of strong selection on low salinity limiting gene flow between populations leading to local adaptation of. A full-factorial common garden experiment with eastern and western *P. microps* being bred and raised in high and low salinity conditions could further our knowledge on how important salinity tolerance is for the fitness of *P. microps* and therefore its dispersal and gene flow between populations.

Roles of natural selection versus genetic drift on phenotypic population divergence in Baltic common gobies: a $P_{ST} - F_{ST}$ comparison

GDMs (using F_{ST} as well as P_{ST}) were not successful to assess the relative importance of IBD versus IBE for population divergence in Baltic common gobies. We therefore decided to take

another approach to investigate the relative contribution of natural selection versus genetic drift causing population divergence in common gobies by using a P_{ST} - F_{ST} comparison. Comparing phenotypic differentiation (P_{ST}) using body shape as trait, to neutral genetic differentiation (F_{ST}) of male and female Baltic common gobies separately, we found for males as well as females that $P_{ST} > F_{ST}$ is clearly larger for a wide range of between and within population additive genetic variances. Such strong results on higher phenotypic than genetic differentiation with non-overlapping 95% confidence intervals suggest that adaptive evolution, i.e. divergent selection on body shape occurs and favours different phenotypes in different Baltic populations. Our results possibly support similar evidence from studies on other fish species (Araujo et al. 2014) showing that salinity can affect body size, and that it is therefore important to include processes governing body size changes when investigating phenotypic population divergence (Collin and Fumagalli 2015).

Pitfalls of using P_{ST} as an approximation of Q_{ST} are well understood and highly criticized (Brommer 2011). Estimating Q_{ST} requires common garden experiments, which quickly become labour-intensive when dealing with multiple populations. Furthermore, these experiments require individuals to be bred and raised in the laboratory, which is very problematic (at best) in the common goby. Consequently, estimates of Q_{ST} are often replaced by the phenotypic analogue, P_{ST} (Leinonen et al. 2008; Leinonen et al. 2013). However, it is generally not recommended to simplify Q_{ST} by its phenotypic analogue P_{ST} , because it is difficult to get the picture of additive genetic variance right when investigating only phenotypic variance. Yet, Leinonen et al. (2008) reviewed several studies, which show no differences in the mean estimates of P_{ST} and Q_{ST} . We, therefore, think that when interpreted with caution P_{ST} - F_{ST} comparisons can provide information about the relative influence of adaptation evolution (natural selection) and neutral evolution (genetic drift) as causes of population differentiation.

Another pitfall, which is not considered conducting a P_{ST} - F_{ST} comparison is the possibility that $P_{ST} > F_{ST}$ is not caused by adaptive genetic divergence but rather by phenotypic plasticity (reviewed in Crispo 2008). Plastic responses (within one generation) are acting much more rapidly on adaptation than natural selection (across generations). However, these two ways of responding to local environmental conditions are not always completely independent of each other; on the contrary, several studies showed that plasticity may drive initial phenotypic divergence followed by genetic changes in the direction of the plastic response (Price et al. 2003; Crispo 2007; Ghalambor et al. 2007). In our study we cannot disentangle if phenotypic divergence in body shape of Baltic *P. microps* is a plastic or an adaptive (genetic) response.

Conclusion

Understanding how and why populations diverge is a fundamental goal of evolutionary biology. Here, we contribute to a growing pool of literature demonstrating that limited gene flow and natural selection in marine environments can cause clear genetic structuring on a fine spatial scale. Furthermore, the relative contribution of adaptive versus neutral evolutionary processes driving population divergence can vary spatially; and by comparing phenotypic differentiation (P_{ST}) to genetic differentiation (F_{ST}) we include another approach on how to investigate the relative contribution of natural selection versus genetic drift in driving phenotypic population divergence. We find IBD as well as IBE to drive population divergence in common gobies, however, to really understand if salinity is the environmental variable responsible for reduced gene flow leading to such a clear genetic structure - especially in Baltic *P. microps* - one would need to conduct full-factorial common garden experiments. With such experiments one could (1) determine Q_{ST} and calculate the actual difference to P_{ST} , and (2) investigate if common gobies are genetically adapted to salinity or just highly plastic

in their response, which would give further insights on their fitness related to dispersal and therefore gene flow between populations.

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Supplemental data

Table S1: Summary statistics for microsatellite data (including *Pmic02*) arranged by locus for each population. Reported are: Sample size (N), number of alleles (N_a), allelic richness (A_r) as well as expected and observed heterozygosity (H_E and H_O). See Table 3 for site abbreviations.

		Pmin35	Pmin38	Pmin04	Pmin05	Pmin09	Pmar03	Pmar05	Pmic02	Pmic03	Mean
KR	N	20	20	20	20	18	19	20	20	20	19.63
	N_a	11	4	2	4	12	12	13	14	6	8.00
	A_r	9.15	3.76	1.89	2.98	9.09	9.16	8.23	10.05	4.50	6.09
	H_E	0.86	0.66	0.14	0.30	0.83	0.80	0.80	0.85	0.66	0.63
	H_O	0.85	0.75	0.15	0.30	0.61	0.79	0.70	0.80	0.853	0.59
KE	N	19	19	19	19	19	19	19	19	19	19
	N_a	10	5	2	5	15	12	11	14	6	8.25
	A_r	8.30	4.51	1.53	3.62	10.33	8.37	8.19	9.64	4.58	6.18
	H_E	0.83	0.74	0.05	0.28	0.87	0.72	0.80	0.84	0.68	0.62
	H_O	0.84	0.84	0.05	0.32	0.84	0.84	0.68	0.84	0.843	0.63
VR	N	10	10	10	10	10	10	10	10	10	10
	N_a	6	4	2	4	6	8	8	13	6	5.50
	A_r	6.00	4.00	2.00	4.00	6.00	8.00	8.00	13.00	6.00	5.50
	H_E	0.80	0.67	0.18	0.47	0.73	0.75	0.74	0.90	0.64	0.62
	H_O	0.50	0.40	0.20	0.60	0.70	0.80	0.90	0.90	0.60	0.59
MA	N	23	23	23	22	20	23	23	23	23	22.50
	N_a	14	4	2	4	13	12	8	13	5	7.75
	A_r	9.81	3.69	1.91	3.73	8.78	8.04	5.25	7.9	4.25	5.68
	H_E	0.88	0.69	0.16	0.45	0.77	0.75	0.65	0.78	0.60	0.62
	H_O	0.83	0.83	0.17	0.45	0.80	0.74	0.83	0.65	0.70	0.67
NE	N	23	23	23	23	21	23	22	19	23	22.63
	N_a	10	4	3	10	10	14	12	16	7	8.75
	A_r	7.80	3.91	2.26	6.17	6.97	8.35	8.89	10.6	4.74	6.13
	H_E	0.84	0.69	0.16	0.50	0.75	0.70	0.86	0.87	0.67	0.65
	H_O	1.00	0.70	0.17	0.52	0.71	0.57	0.68	0.84	0.74	0.64
IP	N	24	24	24	24	24	24	24	24	24	24
	N_a	12	5	2	6	14	14	11	15	6	8.75
	A_r	8.77	4.36	1.81	4.14	9.26	7.86	8.15	9.77	4.22	6.07
	H_E	0.85	0.72	0.12	0.55	0.84	0.68	0.82	0.87	0.56	0.64
	H_O	0.79	0.88	0.13	0.54	0.83	0.58	0.83	0.75	0.63	0.65
PU	N	23	23	23	23	21	23	23	23	23	22.75
	N_a	10	6	2	7	12	14	14	13	5	8.75
	A_r	7.26	4.86	1.83	4.84	8.25	9.33	9.08	7.51	3.95	6.17

Chapter II

	H_E	0.79	0.76	0.12	0.44	0.83	0.83	0.81	0.75	0.54	0.64
	H_O	0.74	0.70	0.13	0.48	0.86	0.78	0.83	0.83	0.52	0.63
KA	N	24	24	24	24	23	24	24	23	24	23.88
	N_a	7	5	2	6	7	11	5	17	5	6
	A_r	5.15	3.15	1.94	3.48	5.83	6.68	3.96	10.93	3.70	4.23
	H_E	0.71	0.26	0.19	0.26	0.71	0.59	0.47	0.89	0.41	0.45
	H_O	0.58	0.25	0.13	0.29	0.65	0.58	0.50	0.87	0.46	0.43
VA	N	22	22	22	22	21	21	22	22	22	21.75
	N_a	9	5	2	5	4	5	4	18	6	5
	A_r	7.33	3.60	1.98	4.07	3.46	3.65	3.70	12.36	4.36	4.02
	H_E	0.80	0.38	0.24	0.42	0.63	0.37	0.64	0.92	0.70	0.52
	H_O	0.73	0.41	0.27	0.45	0.76	0.33	0.73	0.95	0.64	0.54
TH	N	21	20	21	21	20	21	21	19	20	20.63
	N_a	12	4	2	5	7	11	6	16	4	6.38
	A_r	9.26	3.00	2.00	3.67	5.62	6.28	4.40	10.89	3.68	4.74
	H_E	0.86	0.45	0.36	0.30	0.69	0.58	0.37	0.87	0.45	0.51
	H_O	0.86	0.55	0.48	0.29	0.55	0.67	0.43	0.79	0.40	0.53
TV	N	17	17	17	17	17	17	17	15	17	17
	N_a	13	5	2	6	6	9	5	15	2	6
	A_r	10.31	4.01	1.99	5.14	5.01	7.30	4.01	11.89	2.00	4.97
	H_E	0.86	0.47	0.25	0.52	0.65	0.71	0.49	0.90	0.29	0.53
	H_O	0.88	0.41	0.18	0.59	0.53	0.76	0.47	0.80	0.35	0.52
BR	N	24	24	24	24	21	24	24	24	24	23.63
	N_a	13	4	3	10	15	16	14	29	8	10.38
	A_r	9.26	3.98	2.33	6.37	9.93	10.57	8.83	14.92	6.22	7.19
	H_E	0.84	0.72	0.16	0.67	0.83	0.86	0.80	0.93	0.69	0.70
	H_O	0.92	0.63	0.17	0.67	0.67	0.92	0.79	0.96	0.75	0.69
MI	N	15	15	15	15	11	15	14	15	15	14.38
	N_a	12	4	2	10	14	11	9	18	10	9
	A_r	9.88	3.99	1.67	8.60	12.91	9.22	7.00	13.58	8.19	7.68
	H_E	0.84	0.69	0.06	0.79	0.87	0.82	0.71	0.91	0.82	0.70
	H_O	0.87	0.87	0.07	0.80	1.00	0.87	0.64	0.93	0.87	0.75
LV	N	22	21	22	22	15	22	22	21	22	21
	N_a	6	3	2	4	10	13	4	14	3	5.63
	A_r	4.52	2.97	1.85	3.96	7.97	7.76	3.16	10.08	2.99	4.40
	H_E	0.51	0.58	0.13	0.71	0.81	0.76	0.53	0.88	0.62	0.58
	H_O	0.32	0.52	0.14	0.59	0.87	0.91	0.36	0.86	0.64	0.54

CHAPTER III

Gonadal Investment in Common Gobies along the Salinity Gradient in the Baltic Sea

I. M. Mück, J. Stauss, K.U. Heubel

Abstract

Gonadal investment in males using alternative reproductive tactics (ARTs) has been studied extensively in relation to morphological attributes of males, such as body size, as well as demographic and social features, such as population density and mating systems. Although theoretical models suggest a great impact of divergent environments on the fitness of tactics, few studies have considered the effects of the abiotic environment on gonadal investment. Here, we examined gonadal investment (testes and sperm duct gland (SDG) mass) and energy stores (liver mass) in a small marine fish (*Pomatoschistus microps*) along a salinity gradient in the Baltic Sea. Gonad and liver mass of 'bourgeois' males (i.e., nest holding males guarding nests with eggs) and males of a random population sample (RPS) were collected from high, intermediate and low salinity sites. A third group of males, mimicking females, was detected during dissection; this is the first description of female mimics in this species. We found that SDG- and liver mass in nest holder and RPS males was significantly higher at low salinity sites, while testes mass seemed to be unaffected by salinity. Female mimics showed significantly larger testes but smaller SDGs than nest holder and RPS males. Our results contribute valuable information on gonadal investment in a species facing high environmental divergence along a salinity gradient and highlight the importance of including environmental heterogeneity when studying gonadal investment and ultimately ARTs.

Introduction

The influence of the abiotic environment on sexual selection in the form of sperm competition (= when sperm of two or more males compete for the fertilization of a single set of ova; Parker 1970) has largely gone unexamined, and so has its influence on alternative reproductive tactics (ARTs; but see Emlen 2008). The concept of ARTs refers to alternative ways to obtain fertilization of individuals within a population and in spite of theoretical models that suggest a role of the abiotic environment in the evolution of ARTs (Hazel et al. 1990; Gross 1996; Hazel et al. 2004), few empirical studies examined the role of environmental heterogeneity in ARTs (Kolluru and Grether 2005; Lukasik et al. 2006; Larison 2007). Nevertheless, many studies focus on demographic and social features, such as mating systems (Harcourt et al. 1981; Heske and Ostfeld 1990; Sachser et al. 1999; Dunn et al. 2001) and population density (Brown and Brown 2003; Tomkins and Brown 2004; Dziminski et al. 2010) to explain differences in mating tactics and the associated evolution of gonad size (but see also Jivoff 1997; Elgee et al. 2010).

Larger males are often more successful than smaller males in monopolizing territories and females (reviewed in Andersson 1994). To this end, fish are exceptionally well studied. Many species exhibit condition-dependent ARTs, where males switch between being territorial ('bourgeois' males) and sneak mating ('parasitic' males) often depending on male's body size (Magnhagen and Kvarnemo 1989; Rowe et al. 1991; Sato et al. 2004). ARTs are often associated with morphological attributes of different male morphs following different mating tactics (Rasotto and Mazzoldi 2002; Schütz et al. 2010; Utne-Palm et al. 2015). It often remains unclear, however, to what extent ARTs are not only (morphologically) condition-dependent but also environmental-dependent (but see Lukasik et al. 2006; Larison 2007).

The two most energy demanding processes for fish are reproduction and growth. The necessary energy needed for both physiological processes is stored in the liver. Liver mass can

be used as a measure of spawner quality (Marshall et al. 1999) and measuring lipid content allows to estimate the level of energy reserves stored (but see: Lambert and Dutil 1997; Dahle et al. 2003; Sopinka et al. 2009). The distribution of energy reserves can vary markedly with gender (Casselman and Schulte-Hostedde 2004), season (Elofsson et al. 2003; Resende et al. 2005) and food abundance (Rideout et al. 2004). A drop in energy reserves in three-spine sticklebacks just before the start of the breeding season suggests a possible direct effect in investing energy stored in the liver into gonads (Chellappa et al. 1989; but see: Chellappa et al. 1995; Malavasi et al. 2004; see for Gobiidae: Fiorin et al. 2007). Reproductive investment and energy reserve storage as well as replenishment can vary markedly among individuals (Fiorin et al. 2007) and whether an individual reproduces depends mostly on body size and condition (Rowe et al. 1991; Simpson 1992; Adams and Huntingford 1997; Hutchings et al. 1999). Overall, body size in fish is strongly correlated with a male's mating tactic, the size of its gonads, and the level of energy reserves it has stored in the liver. Thus, differences in body size need to be considered when investigating gonadal and liver investment among populations (Tomkins and Simmons 2002; Stoltz et al. 2005).

Generally, independent of environmental aspects, reproductive resource-controlling males face a lower risk of sperm competition compared to sneak mating opportunistic males (Parker et al. 1997). In the bluegill sunfish (*Lepomis macrochirus*), for example, bourgeois males experience sperm competition in about 10% of their mating events, while sneaker males always experience sperm competition, because the bourgeois males are always present (Neff et al. 2003; Ota and Kohda 2006). Therefore, one would expect a trade-off between male types and their reproductive investment in for instance mate attraction, intra-sexual competition (bourgeois males), or just predominately in gonads (sneaker males; Immler et al. 2004). Sneaker males are expected to have larger testes producing a higher ejaculate volume than

bourgeois males (Kvarnemo et al. 2010); conversely, the opposite is expected for their sperm-duct glands (SDGs) due to a trade-off with testes investment. SDGs (also referred to as accessory glands or seminal vesicles, e.g. Fishelson 1991) are specialized accessory organs near the testes producing sperm-containing mucus (sperm-trails), which nest-holding males use to cover the inner surface of their nest to possibly enhance egg survival (Giacomello et al. 2006).

Research on gonadal investment has tended to focus on morphologically different male morphs using ARTs. There is a lack of knowledge on how the abiotic environment influences the fitness of tactics and the risk and intensity of sperm competition, which in turn affects gonadal investment and ultimately the fitness consequences of ARTs. The goal of this study was to relate gonadal investment and energy storage in fish using ARTs to an abiotic environmental factor that strongly varies among populations. More precisely, we examined how salinity in the Baltic Sea (decreasing from West to East) co-varies with gonad- and liver mass of the common goby (*Pomatoschistus microps*). In an earlier study (Chapter I) we investigated the geographic variation of abiotic and biotic parameters of common goby populations throughout the Baltic Sea. Results showed that salinity influences the whole common goby-system in the Baltic Sea - both directly (body size, population densities, brood characteristics) and indirectly (nest quantity and quality). We collected a random population sample (RPS) of common goby males and females of high, intermediate and low salinity sites, as well as specifically nest holding (NH) males guarding eggs. Nest holding males are the 'bourgeois' males of the population (Magnhagen 1994), and we only make predictions for this male category. We expected to find the largest testes in NH males of intermediate salinity sites as a result of larger population densities at these sites (see Chapter I; Brown and Brown 2003), increasing the intensity of sperm competition. We predict to find the largest SDGs in

NH of low salinity sites. Low salinity was shown to bear a higher risk of egg infection (Lehtonen and Kvarnemo 2015a) and possibly decreases sperm motility (Elofsson et al. 2003), thus large SDGs producing large amounts of protective mucus are advantageous. Energy storage estimated as liver mass, should generally be lower in bourgeois males (i.e., NH males) due to reproductive investment and defending and caring for eggs (Malavasi et al. 2004). In addition, we expected higher liver mass in bourgeois males at low salinity sites than at high salinity sites, because living in low-salinity conditions may cause high costs of osmoregulation requiring large energy stores (i.e., liver mass) for a marine species as the common goby (Tseng and Hwang 2008) .

Although we mainly focused on males, we also analysed females to make sure that we did not overlook any males not displaying obvious male characteristics. This way we were also able to compare gonad investment and liver mass of females (no ART-specific adaptation in females) among populations with patterns of geographic variation in salinity, providing insights into adaptive evolution.

Methods

Study species

The common goby (*Pomatoschistus microps*; Fig. 2) is an annual, benthic fish that occurs along the European Atlantic coast, including the Baltic Sea. Common gobies have a promiscuous mating system reproducing several times during a single reproductive season (Miller 1975). Common goby males were shown to adopt different reproductive tactics, with larger males defending nests and providing parental care ('nest holder') and smaller males sneaking fertilization ('sneaker') (Magnhagen 1992; Magnhagen 1994). Males take up nests under

empty mussel-shells or rocks and court females to lay eggs inside the nest; thereafter the male provides uni-parental care till the eggs hatch (Magnhagen and Vestergaard 1993).

Sample collection

Fish were collected during the breeding season (May-July) in 2014 (one exception: fish of IP collected 2013) from five different populations (KR, KE, IP, GO, TV) along the salinity gradient in the Baltic Sea, categorized into high, intermediate and low salinity sites (see results of Chapter I; see Table 1 for information on sampling sites). Besides females of different ripeness stages (R1: unripe, R2: ripe, R3: ready-to-mate; Fig. 3), RPS (random population sample) males, and nest holding (NH) males, some individuals were categorized during dissection into a third male category, namely 'female mimics' (FMs). These female mimicking males, which lack obvious male characteristics such as the black anal fin or the blue spot on the dorsal fin (Nyman 1953), were initially categorized as females of R1 during collection. Only during dissection (see below for details) it became clear that those alleged females had male gonads. The sample size of FMs per population was however very small (Table 7). All fish collected were measured to the nearest 0.5 mm (Table 7), weighed to the nearest 0.001g and afterwards euthanized and stored in 96% ethanol at - 80°C until dissection.

Chapter III

Table 7. Reported are the number of random population sample (RPS) males, nest holding (NH) males and males mimicking females (FM; N Males) as well as the number of females (N Females) of three different ripeness stages (R1: unripe, R2: ripe and R3: ready-to mate). Furthermore, the mean body length [mm TL] \pm SE (standard error) is reported for each male and female category for the different sampling sites (for N \leq 2 the absolute values for body length are given).

Sampling site	Abbr.	N Males			N Females			Total length [mm]						
		RSP	NH	FM	R1	R2	R3	total	RSP	NH	FM	F (R1)	F (R2)	F (R3)
Kristineberg	KR	52	21	3	28	27	10	65	39.3 \pm 0.34	40.7 \pm 0.45	36.3 \pm 0.33	38.9 \pm 0.55	38.1 \pm 0.73	38.1 \pm 1.18
Kerteminde	KE	57	23	6	5	22	3	30	37.2 \pm 0.48	38.2 \pm 0.54	30.8 \pm 1.86	32.9 \pm 1.62	36.3 \pm 0.58	36.7 \pm 1.45
Island Poel	IP	25	18	2	2	21	0	23	35.7 \pm 0.49	36.1 \pm 0.51	30.0, 30.0	34.0, 31.0	35.7 \pm 0.50	----
Gotland	GO	26	26	0	2	3	22	26	35.1 \pm 0.64	35.1 \pm 0.47	----	35.0, 34.0	36.0 \pm 0.24	36.2 \pm 0.75
Tvärminne	TV	31	25	0	0	28	2	30	33.9 \pm 0.52	35.2 \pm 0.73	----	----	34.9 \pm 0.41	35.5, 38.5

Fish dissection

First all males, thereafter all females (including FMs) were dissected in a random order. To do so, fish were taken out of the ethanol and gently dried with a paper towel. With a medial ventral cut the

abdominal cavity of the fish was opened and liver, gonads and gut (which was discarded and not used for analyses) were removed.

Gonads of male common gobies are composed of the testes as well as SDGs (see Fig. 15), which were removed and treated separately.

Gonads of females are referred to as ovaries

Box 1. The down-side of the Gonado-Somatic-Index (GSI)

Knowing from previous studies that *P. microps* has evolved condition-dependent alternative mating tactics (Magnhagen 1992; Magnhagen 1998) as well as that body size decreases from West to East in the Baltic Sea (see Chapter I) we decided against the highly criticized (Tomkins and Simmons 2002; Stoltz et al. 2005) but still very often used method of calculating the gonado somatic index ($GSI = 100 \times \text{gonad mass} / \text{total body mass}$; Hutchings and Myers 1994; Taborsky 1998). Ratios like the GSI or HSI (hepatosomatic-index) do not properly account for underlying body weight variation. Any changes in gonad or liver mass depending on different salinity levels, male category, or female ripeness stage may come about by body size differences due to allometric scaling. The theory of allometry implies that if an individual's body size changes (like *P. microps* does along the salinity gradient in the Baltic Sea) so do e.g. its organs – but at different rates. In other words, large individuals have larger organs than small individuals of the same species, however, depending on origin or type, the investment of large or small individuals may be proportionally larger (allometric exponent > 1) or smaller (allometric exponent < 1 ; Tomkins and Simmons 2002; Brockmann 2008; Han and Jablonski 2016). It is therefore necessary to account for intraspecific differences in body size when investigating relative gonad size between populations. If gonad mass would be isometrically related to body size (exponent = 1) one could simply use the GSI or HSI and measure the relative investment into sperm/liver production controlling for differences in body size (Parker 1990). However, isometric relationships appear to be rare in nature (Gould 1966) and especially in fish, therefore using the GSI or HSI as approach to investigate gonadal/liver investment in fish seems to be inappropriate, because ratios do not control for body size due to allometric and not isometric relationships.

(Fig. 16). The eviscerated body (= soma mass), the liver and the gonads of all fish were put into separate reaction tubes and dried in an oven (Binder BG 115) at 60°C for 24 hours. Thereafter body parts were weighed using an electronic analytical balance for soma, female liver and ovaries ($d = 0.0001\text{g}$; Sartorius LE324s) and a microbalance for male liver, testes and SDGs ($d = 0.001\text{mg}$; Sartorius M2P).

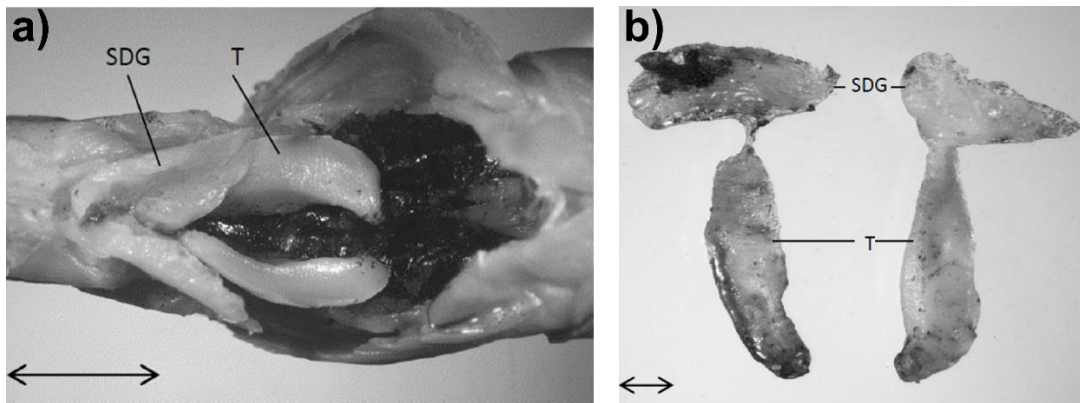


Figure 15. Shown are a) the position and size of male gonads (T: testes and SDG: sperm duct glands, size standard = 0.5 cm) inside the body with gut and liver removed and b) pairwise male gonads (size standard = 1 mm) after being removed from the body.

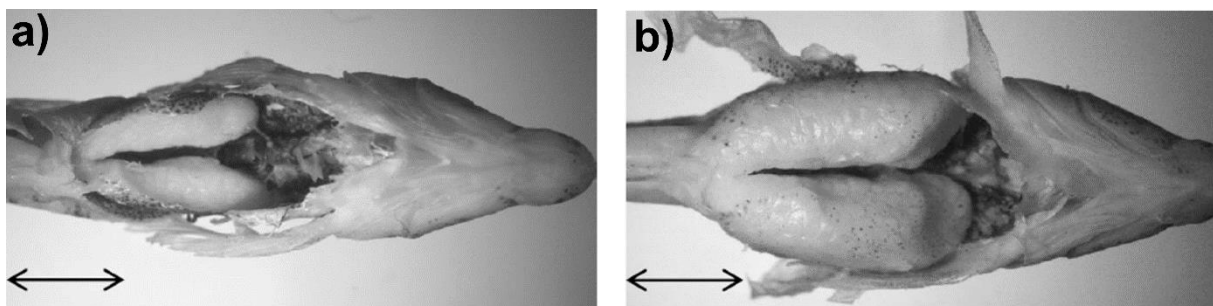


Figure 16. Shown are the position and size of female ovaries inside the body with gut and liver removed (size standard = 0.5 cm). a) Ovaries of a female in ripeness stage 1 (R1) and b) ovaries of a female in ripeness stage 3 (R3).

Data analysis

Based on results of an earlier study (see Chapter I) we divided the five Baltic populations into high, intermediate and low salinity sites (Table 1). However, we compared the AICs (Akaike Information Criterion) of models (see below) including all five populations with the AICs of the model including the three salinity levels and found the simplified models (lower AICs) to show a better fit to the data (Crawley 2005). Therefore, we combined data from the different populations according to salinity levels (high, intermediate, low) for further analyses.

Data for soma mass, testes mass, SDG mass, ovary mass and liver mass were \log_{10} transformed to deal with allometric scaling effects (see Box 1) and to reach normality (Tomkins and

Simmons 2002). Additionally, we calculated the proportion of SDGs of the total gonad mass. To correct for average body size differences between populations we mean centered soma mass after \log_{10} transformation and included it as a covariate. In order to avoid spurious correlations when investigating part-whole relationships, we included soma mass rather than body mass, which would include gonads and liver (Tomkins and Simmons 2002; Stoltz et al. 2005).

We decided against the calculation of the gonado-somatic index (GSI; see Box 1) but used separate linear models (LMs) in R using R Studio (Version 0.98.1091- © 2009-2014 RStudio, Inc.; R Core Team 2012) to examine slope and intercept differences in gonadal and liver investment in males and females following and Tomkins and Simmons (2002) and Stoltz et al. (2005). This approach allows us to evaluate differences in the allometric scaling of gonad and liver mass with soma mass as covariate among salinity levels, male categories, and female ripeness stages (R1 - R3; Neff et al. 2003; Hayward and Gillooly 2011).

Specifically, we investigated how gonadal and liver investment differs between: (I) males (RPS+NH males) originating from populations of three salinity levels with '*salinity*' (high, intermediate, low) and '*male category*' as fixed factor and '*centred log soma mass*' as covariate (model 1), (II) males in a certain role (only NH males) originating from three different salinities and thus '*male category*' not included as a fixed factor (model 2) and (III) males of three different '*male categories*': a random population sample (RPS) of males, males within a certain role (NH males) and males mimicking females (FMs) but without '*salinity*' as fixed factor due to a very low sample size of FMs (see Table 7; model 3). Pooling FMs (N = 11 for testes- and liver mass, N = 10 for SDGs and proportion of SDGs) it was possible to compare FMs with RPS (N = 191) and NH (N = 113) males regarding their gonad mass and liver mass to illustrate the differences. Furthermore, we also investigated how ovary and liver investment

differs between: (IV) females (R1-R3) of different salinity levels with '*salinity*' and '*ripeness*' as fixed factors and '*centred log soma mass*' as covariate (model 4 equivalent to model 1) and (V) females of different ripeness stages removing '*salinity*' as fixed factor (model 5 equivalent to model 3).

Initially, all full models included fixed factors, the covariate and all possible interactions. Model selection in a backward stepwise fashion was performed starting from the full model including all interaction terms towards the final model (Table 8). We conducted comparisons of the estimates of intercepts and slopes between the levels within factors (salinity, male category, female ripeness stage). Reported are always the estimates of the coefficients (*a* for intercepts and *b* for slopes) of the final model and its standard error (\pm SE) for each factor level. Furthermore, t-test results of significant pairwise differences ($P < 0.05$) of intercept (*a*) and slope (*b*) estimates as summary statistics to allow within and across study comparisons (Neff et al. 2003; Stoltz et al. 2005; Ebert et al. 2011). Additionally, we used linear regressions to investigate the relationship between male \log_{10} total gonad mass (testes mass + SDG mass) and liver mass and for females ovary mass and liver mass.

Table 8. Final linear models - providing estimates for intercept and slope interpretation for gonadal- (testes, sperm duct glands (SDG), proportion of SDG of the total gonad mass) and liver-mass variation of the five models (1-5) with different data domains (phenotypic males (RPS+NH), nest holding males (NH), all male categories (RPS+NH+FM) and females (F). Models had different predictors like salinity (high, intermediate, low), male category (RPS, NH, FM) and female ripeness stage (R1-R3). See Table 7 for explanation of abbreviations. Variables that were included in the final model are listed and given in bold when they showed to have a significant effect.

Response Variable	Predictor	Model type	Data domain	adj. R ² (%)	df	F	P	<i>variables included in model</i>
phenotypic males								
Testes	Salinity	1	RPS+NH	46	4, 299	65.3	< 0.0001	salinity+cent.soma+M.category
Liver	Salinity	1	RPS+NH	61	2, 297	81.3	< 0.0001	salinity+cent.soma+M.category+salinity:cent.soma
SDG	Salinity	1	RPS+NH	54	6, 297	60.2	< 0.0001	salinity+cent.soma+M.category+salinity:cent.soma
Prop. SDG	Salinity	1	RPS+NH	45	6, 297	42.8	< 0.0001	salinity+cent.soma+M.category+salinity:cent.soma
subset nest holders								
Testes	Salinity	2	NH	53	3, 109	43.7	< 0.0001	salinity+cent.soma
Liver	Salinity	2	NH	59	5, 107	32.7	< 0.0001	salinity+cent.soma
SDG	Salinity	2	NH	42	3, 107	29.7	< 0.0001	salinity+cent.soma
Prop. SDG	Salinity	2	NH	42	5, 107	17.2	< 0.0001	salinity+cent.soma+salinity:cent.soma
all male categories								
Testes	Male category	3	RPS, NH, FM	42	3, 311	77.9	< 0.0001	M.category+cent.soma
Liver	Male category	3	RPS, NH, FM	41	3, 311	74.7	< 0.0001	M.category+cent.soma
SDG	Male category	3	RPS, NH, FM	35	3, 310	57.6	< 0.0001	M.category+cent.soma
Prop. SDG	Male category	3	RPS, NH, FM	15	3, 310	19.7	< 0.0001	M.category+cent.soma
females								
Ovaries	Salinity	4	F	42	5, 168	25.5	< 0.0001	salinity+cent.soma+ripeness
Liver	Salinity	4	F	69	7, 166	55.9	< 0.0001	salinity+cent.soma+ripeness+salinity:cent.soma
Ovaries	Ripeness	5	F	36	3, 170	33.4	< 0.0001	cent.soma+ripeness
Liver	Ripeness	5	F	35	3, 170	32.4	< 0.0001	cent.soma+ripeness

Results

Males

Differences in gonadal and liver investment between salinity levels

Gonad and liver mass of males exhibiting typical male phenotypes (= RPS+NH males of model 1, hereafter referred to as 'males'; Table 8), and of nest holding males (= NH males of model 2, hereafter referred to as 'NH males'; Table 8) originating from three salinity levels in the Baltic Sea (high, intermediate, low) were investigated. The effect of salinity on gonad and liver mass based on final linear models (Table 8) in relation to '*soma mass*' is presented as separate intercepts and slopes for each response variable (Fig. 17).

Testes

No significant differences in males and the subset of known NH males' testes mass were detected between the three salinity levels (Table 9a, b, Fig. 17a,)

Liver

Liver mass was significantly higher in males and NH males of low than of high or intermediate salinity habitats (Table 9a, b, Fig. 17b). Liver mass of males and NH males originating from high and intermediate salinity sites did not differ. '*Male category*' had an effect on liver mass, with NH males showing a heavier liver (on average 0.90 mg) than males in general.

The slope of the relationship between liver mass and soma mass of males differed only significantly between intermediate ($b = 1.58 \pm 0.11$) and low ($b = 1.18 \pm 0.12$) salinity habitats. There was no significant difference in the slope between high ($b = 1.61 \pm 0.20$) and intermediate and high and low salinity sites (Table 9b).

Table 9. Reported are a) estimates for intercepts [a] and standard error [\pm SE] for each factor level and b) pairwise differences of intercepts [a] and slopes [b] of \log_{10} gonad mass (testes, sperm duct glands (SDGs) and proportion of sperm duct glands of total gonad mass (Prop SDG)) and \log_{10} liver mass of all phenotypically recognisable males (RPS+NH males, model 1) and the subset of NH males (model 2) between three salinity levels (high, intermediate: int., low) of the Baltic Sea and between three male categories (RPS, NH, FM, model 3) independent of salinity level. See Table 7 for explanation of abbreviations and Table 8 for statistical details of underlying final models.

a)

			log Testes		log SDG		Prop SDG		log Liver	
		Model	Intercept [a]	SE	Intercept [a]	SE	Intercept [a]	SE	Intercept [a]	SE
Salinity level	RPS+NH									
	high	1	-0.09	0.02	-0.08	0.04	0.52	0.02	0.63	0.03
	int	1	-0.08	0.01	-0.28	0.02	0.40	0.01	0.60	0.02
	low	1	-0.11	0.01	0.08	0.02	0.61	0.01	0.88	0.01
Salinity level	NH									
	high	2	-0.11	0.03	-0.18	0.04	0.52	0.01	0.57	0.04
	int	2	-0.11	0.02	-0.25	0.03	0.42	0.01	-0.57	0.02
	low	2	-0.09	0.02	0.05	0.03	0.59	0.01	0.87	0.02
Male category										
	RPS	3	-0.09	0.01	-0.14	0.02	0.48	0.01	0.70	0.01
	NH	3	-0.11	0.01	-0.11	0.02	0.50	0.001	0.60	0.02
	FM	3	0.05	0.04	-0.75	0.08	0.16	0.04	0.63	0.06

Chapter III

b)

		log Testes				log SDG				Prop SDG				log Liver			
		Intercept [a]		Slope [b]		Intercept [a]		Slope [b]		Intercept [a]		Slope [b]		Intercept [a]		Slope [b]	
Model		t	P	t	P	t	P	t	P	t	P	t	P	t	P	t	P
Salinity	Data: RPS+NH																
	high-int	1	1.01	0.313		-4.63	< 0.0001	3.85	0.0002	-5.41	< 0.0001	4.13	< 0.0001	-0.83	0.406	-0.16	0.873
	high-low	1	-0.44	0.661		3.74	< 0.0001	1.47	0.143	3.57	0.0004	2.14	0.033	6.5	< 0.0001	-1.86	0.064
	int-low	1	-1.75	0.08		13.1	< 0.0001	-3.4	0.0008	14	< 0.00001	-2.84	0.004	11.75	< 0.0001	-2.47	0.014
Salinity	Data: NH																
	high-int	2	0.08	0.934		-1.54	0.126			-2.65	0.009	1.09	0.275	-0.11	0.91		
	high-low	2	0.55	0.587		4.17	< 0.0001			0.99	0.322	2.44	0.016	5.74	< 0.0001		
	int-low	2	0.7	0.487		-8.19	< 0.0001			8.74	< 0.0001	1.98	0.05	8.62	< 0.0001		
Male category	RPS, NH, FM																
	RPS-NH	3	-0.49	0.62		1.1	0.274			1.15	0.252			-0.2	0.844		
	RPS-FM	3	3.63	0.0003		-7.44	< 0.0001			-7.06	< 0.0001			-1.03	0.306		
	NH-FM	3	3.74	0.0002		-7.69	< 0.0001			-7.35	< 0.0001			-0.94	0.351		

Sperm duct glands (SDGs)

Males and NH males of low salinity habitats had significantly larger SDGs than males originating from intermediate or high salinity habitats (Table 9a, b, Fig.17c). Males but not NH males had significantly larger SDGs in high than in intermediate salinity habitats (Fig 17c).

The slope of the relationship between SDG mass and soma mass was significantly steeper at intermediate salinity ($b = 1.61 \pm 0.13$) than under high ($b = 0.56 \pm 0.24$) and low ($b = 0.96 \pm 0.14$) salinity conditions (Table 9b). The slope did not differ between high and low salinity levels.

Proportion of sperm duct glands (SDGs) of total gonad mass

Also the proportion of SDGs of the total gonad mass was highest in males and NH males of low salinity habitats (~ 60 %) and differed significantly to intermediate and high (only in males) salinity sites (Table 9a, b, Fig. 17d).

The slope of the relationship between the proportion of SDGs and soma mass differed in males significantly between all three salinity levels with a positive slope for low ($b = 0.15 \pm 0.07$) and intermediate salinity ($b = 0.44 \pm 0.07$) and a negative slope for the high salinity level ($b = - 0.17 \pm 0.13$; Table 9); for NH males the slope was positive and significantly steeper for low ($b = 0.23 \pm 0.09$) salinity habitats compared to a strong negative slope for the high ($b = - 0.43 \pm 0.25$) salinity site but no difference in slopes between high and intermediate, and low and intermediate ($b = - 0.11 \pm 0.14$) salinity sites were detected (Table 9b, Fig. 17d).

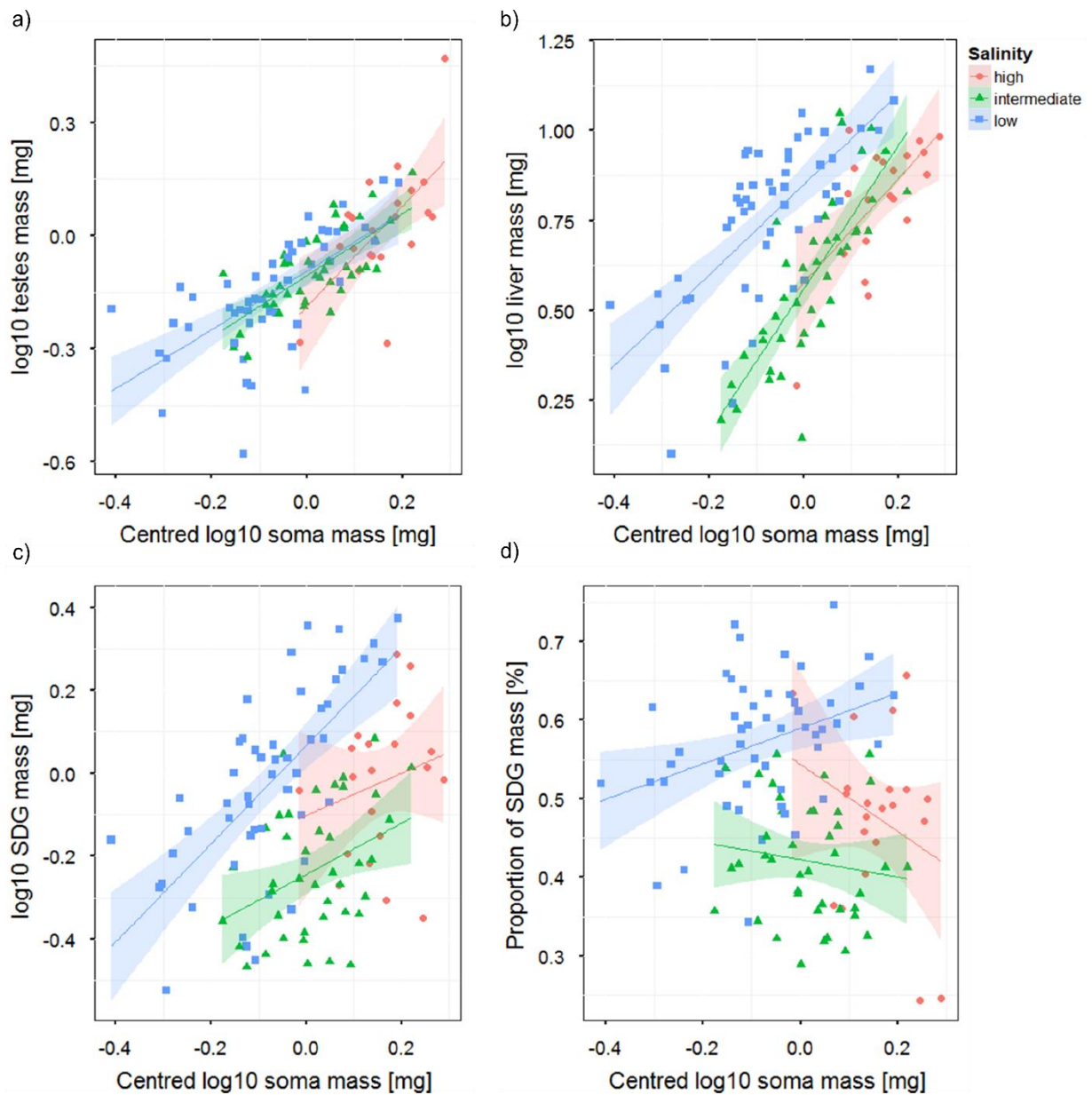


Figure 17. Results of linear models on gonadal and liver investment at different salinity levels (high: red rhomb, intermediate: green triangle and low: blue square) in nest holding (NH) males (model 2). Log₁₀ a) testes mass, b) liver mass, c) sperm duct gland (SDG) mass and the d) proportion of SDGs of the total gonad mass (Y-axis) regressed on to centred log₁₀ soma weight (X-axis). Graphs of all phenotypically recognisable males (model 1) are not shown as the patterns look almost identical to the plots presented here. Shading shows 95% confidence intervals.

Differences in gonadal and liver investment between male categories

Irrespective of variation in salinity, gonad and liver mass of three male categories (RPS, NH, FM, model 3) were analysed (Table 8). This was done because among 106 individuals of KR (high salinity) categorised as females three males mimicking females (FMs) were detected during dissection. Also at the intermediate salinity site KE out of 97 individuals *a priori* defined as females six FMs were found and at the intermediate site IP out of 25 allegedly defined females two FMs were found during dissection. No FMs were detected among females of the low salinity sites GO and TV (see Table 1 for site abbreviations). The effect of 'male category' on gonad and liver mass based on final linear models (Table 8) in relation to 'soma mass' is presented as separate intercepts and slopes for each response variable (Fig. 18).

Testes

Testes mass of FMs (1.1 ± 0.4 mg) was significantly higher than of RPS (0.9 ± 0.02 mg) and NH (0.8 ± 0.03 mg) males (Table 9a, Fig. 18a). Testes mass did not differ between RPS and NH males (Table 9b).

Liver

No significant differences between the three male categories (FM: 3.1 ± 0.5 ; RPS: 5.9 ± 0.2 mg; NH: 5.8 ± 0.3 mg) were detected in liver mass (Table 9a, b, Fig. 18b).

Sperm duct glands (SGDs)

Results on SDG mass showed exactly the opposite than for testes mass with SDGs of FMs (0.16 ± 0.05 mg) being significantly smaller than of RPS (0.90 ± 0.004 mg) and NH (0.89 ± 0.04 mg) males (Table 9a, Fig. 18c). SDG mass of RPS and NH males did not significantly differ (Table 9b).

Proportion of sperm duct glands (SDGs) of total gonad mass

Analog to results on absolute sperm duct gland mass, also the proportion of SDGs of the total gonad mass was significantly lower in FMs (0.16 ± 0.03) than in RPS (0.50 ± 0.01) and NH (0.48 ± 0.01) males (Table 9, Fig. 18d). There was also no significant difference in the proportion of SDGs of the total gonad mass between RPS and NH males (Table 9b).

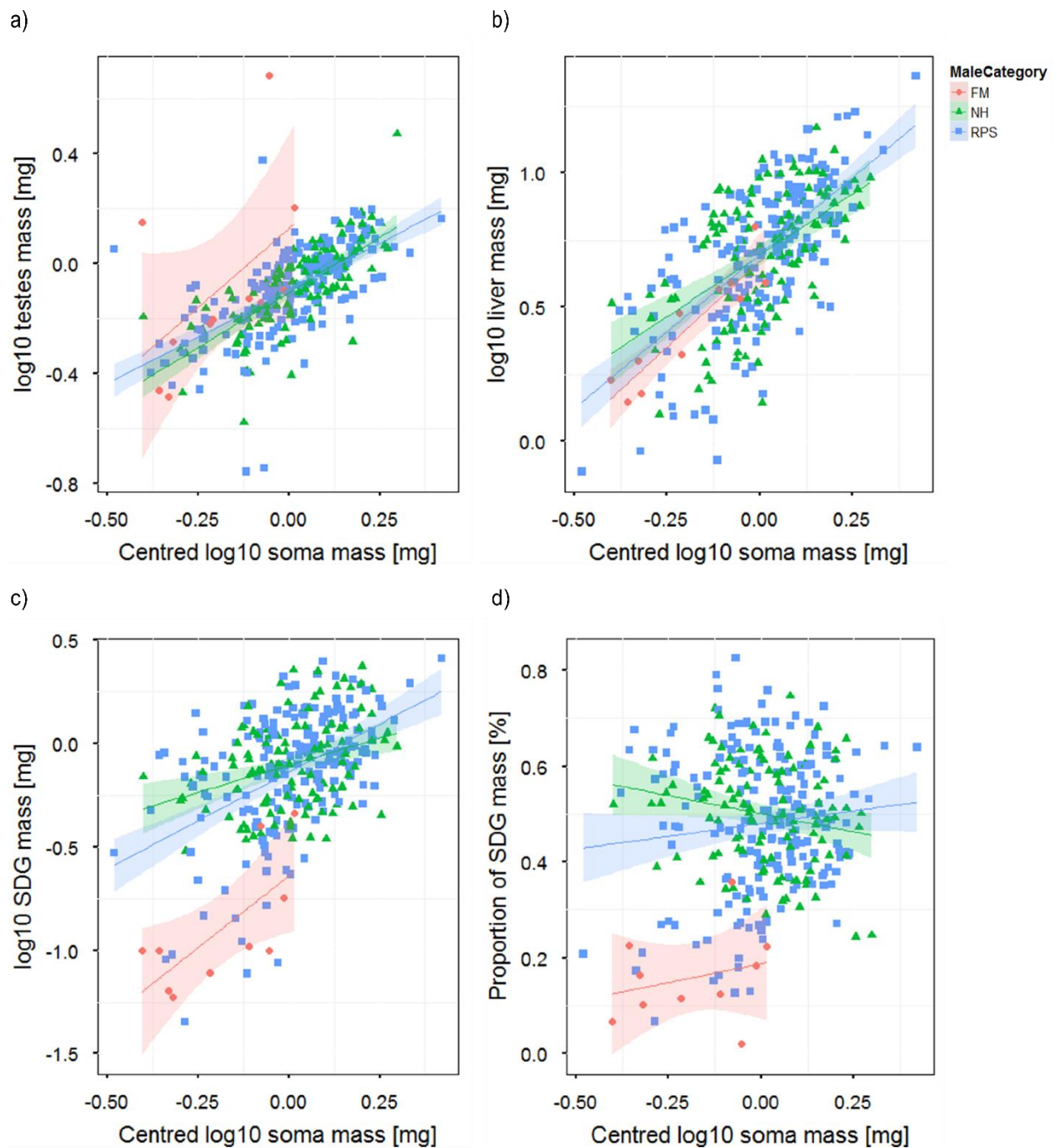


Figure 18. Results of model (3) with \log_{10} a) testes mass, b) liver mass, c) sperm duct gland (SDG) mass and the d) proportion of SDGs of total gonad mass (Y-axis) regressed on to centred \log_{10} soma mass (X-axis) of FM males (rhomb, red), NH males (triangle, green) and RPS males (square, blue). Shading shows 95% confidence intervals. See Table 7 for explanation of abbreviations of male categories.

Females

Ovary and liver mass of common goby females originating from three salinity levels in the Baltic Sea were analysed (model 4). Moreover, differences in ovary and liver mass between females of the different ripeness stages (R1-R3) were analysed to test the validity of the visual categorization into the three ripeness stages *a priori* (model 5). The effect of salinity and ripeness stage on gonad and liver mass based on final linear models (Table 8) in relation to 'soma mass' is presented as separate intercepts and slopes for each response variable (Fig. 19).

Differences in gonadal and liver investment between salinity levels

Ovaries

Ovary mass is dependent on the ripeness stage of a female (see Fig. 19c). The number of females in different ripeness stages (R1-R3) varied among salinity levels (see Table 1); consequently comparing ripeness stages between salinity levels is not informative (but see Table 3 and 8; Fig. 19a).

Liver

Liver mass was significantly higher in females of low than intermediate or high salinity habitats (Table 10a, b, Fig 19b). Liver mass was lowest in high salinity sites and differed significantly from that in intermediate salinity sites.

The slope of the relationship between liver mass and soma mass differed significantly between females of high ($b = 1.25 \pm 0.11$) and low ($b = -0.86 \pm 0.12$) salinity habitats. The slope of intermediate salinity ($b = 1.17 \pm 0.12$) did not differ from high or low salinity habitats (Table 10 b).

Table 10. Reported are a) estimates for intercepts [α] and standard error [\pm SE] for each factor level and b) pairwise differences of intercepts [α] and slopes [b] of \log_{10} ovaries and \log_{10} liver mass of females originating from three different salinity levels (high, intermediate, low; model 4) and between three different ripeness stages (R1-R3; model 5). See Table 7 for explanation of abbreviations and Table 8 for statistical details on underlying final models.

a)

		log Ovaries			log Liver	
		Model	Intercept [α]	SE	Intercept [α]	SE
Salinity level						
	high	4	0.90	0.05	0.53	0.02
	int	4	0.70	0.06	0.60	0.02
	low	4	0.92	0.07	0.79	0.02
Ripeness stage						
	R1	5	0.86	0.05	0.58	0.02
	R2	5	1.17	0.03	0.66	0.01
	R3	5	1.49	0.05	0.65	0.02

b)

		log Ovaries				log Liver				
		Intercept [α]			Slope [b]		Intercept [α]		Slope [b]	
		Model	t	P	t	P	t	P	t	P
Salinity level										
	high-int	4	-3.43	0.0008			3.3	0.001	-0.5	0.611
	high-low	4	0.29	0.773			12.3	< 0.0001	-2.45	0.015
	int-low	4	3.74	0.0003			9.13	< 0.0001	-1.86	0.063
Ripeness stage										
	R1-R2	5	5.32	< 0.0001			3.31	0.001		
	R1-R3	5	8.97	< 0.0001			2.06	0.041		
	R2-R3	5	-5.37	< 0.0001			-3.22	0.03		

Differences in gonadal and liver investment between female ripeness stages

Ovaries

Ovary mass differed significantly between the three ripeness stages (R1-R3). Females of R3 had significantly heavier ovaries (34.5 ± 2.1 mg) than females of R2 (18.2 ± 1.0 mg); and ovaries of R2 females were heavier than those of R1 (9.9 ± 1.1 mg) females (Table 10a, b, Fig. 19c).

The slope of the relationship between ovary mass and soma mass did not differ significantly between female ripeness stages (Table 10b).

Liver

The three ripeness stage of females had an effect on liver mass. Females of R1 (4.3 ± 0.3 mg) had significantly lower liver mass than females of R2 (4.8 ± 0.2 mg) and R3 (4.9 ± 0.3 mg; Table 10a, b, Fig. 19d). Females of R2 and R3 did not significantly differ in liver mass.

The slope of the relationship between liver mass and soma mass did not differ between female ripeness stages (Table 10b).

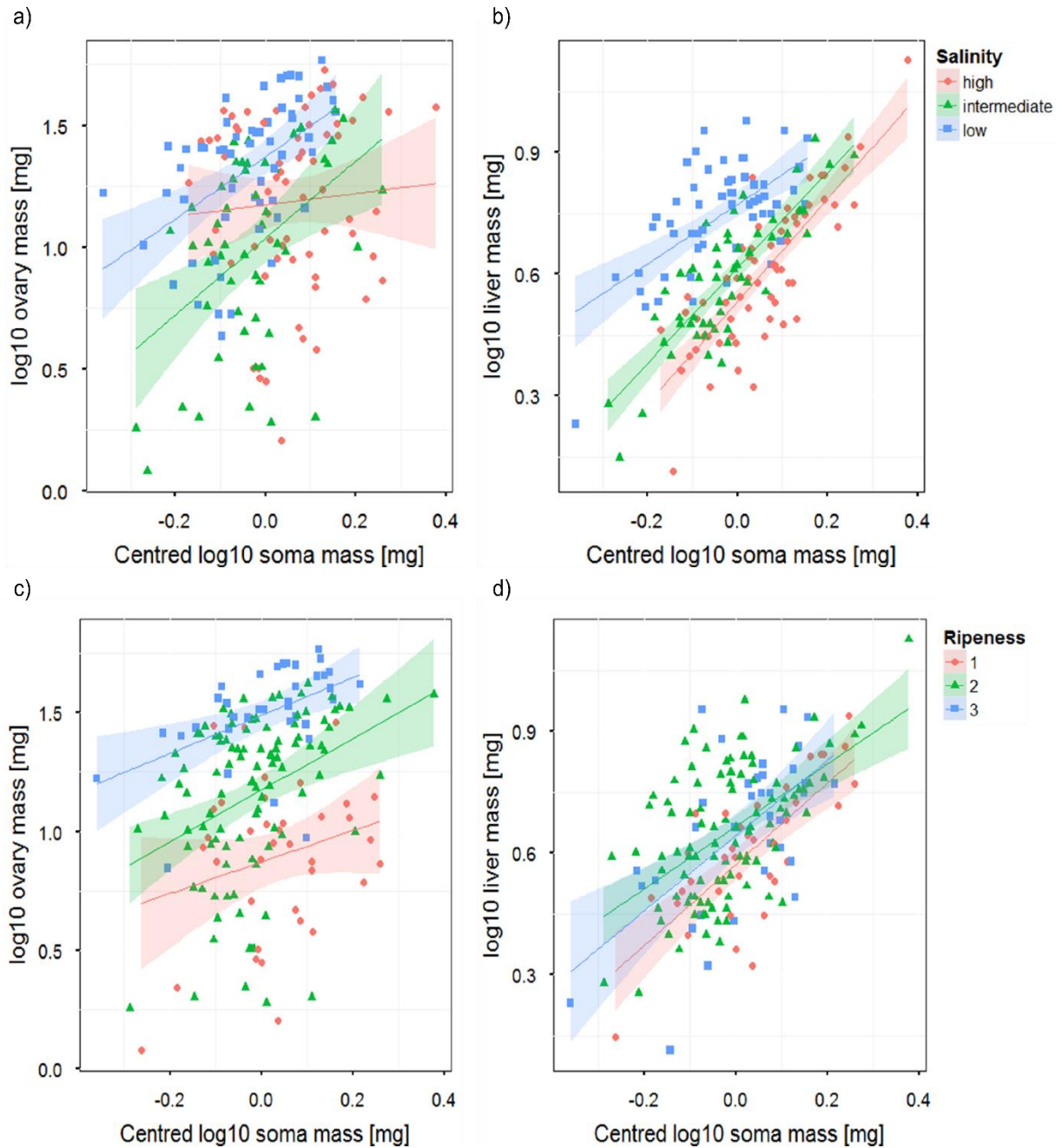


Figure 19. Graphs show results of model 4 with log₁₀ a) ovary mass and b) liver mass of females (Y-axis) originating from high (rhomb, red), intermediate (triangle, green) and low (square, blue) salinity habitats regressed on to centred log₁₀ soma mass (X-axis) and results of model 5 with log₁₀ c) ovary mass and d) liver mass of females (Y-axis) categorised in three ripeness stages: R1 (rhomb, red), R2 (triangle, green) and R3 (square, blue) regressed on to centred log₁₀ soma mass (X-axis). Shading shows 95% confidence intervals. See Table 7 for explanation of abbreviations of ripeness stages.

Gonad and liver mass

There was a significant relationship between the total gonad mass (testes mass + SDG mass = total gonad mass) and liver mass in males (RPS+NH+FM) with a correlation coefficient of 0.60 (linear regression: $R^2 = 36\%$, $F_{1,302} = 170.0$, $P < 0.0001$; Fig. 20a). In females (only R2 and R3 included) the relationship of ovary mass and liver mass was less clear with a correlation coefficient of 0.25 (linear regression: $R^2 = 6\%$, $F_{1,135} = 9.4$, $P = 0.0003$; Fig. 20b).

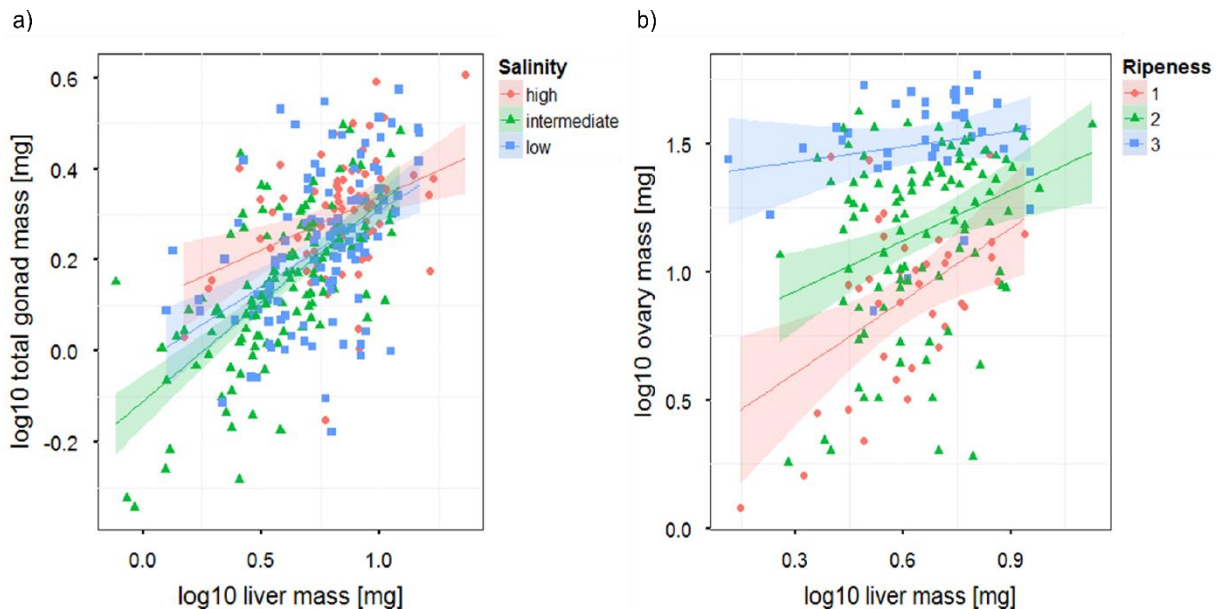


Figure 20. The relationship (linear regression) of a) log₁₀ total gonad mass (= testes mass + SDG mass) and log₁₀ liver mass of males originating from three different salinity habitats (high, intermediate, low) as well as the relationship of b) log₁₀ ovary mass and log₁₀ liver mass of females in three different ripeness (R1- R3) stages. Shading shows 95% confidence intervals. See Table 7 for explanation of abbreviations of ripeness stages.

Discussion

The main findings of this study were (1) the first discovery of male common gobies mimicking females and that these female mimics had significantly larger testes but smaller SDGs than other males, (2) that males of low salinity sites had larger SDGs, but testes size seemed not to be affected by salinity and (3) that individuals (males and females) had larger energy stores (i.e., liver mass) at low salinity sites.

Female mimics in common gobies

Here, we describe for the first time in common gobies that 'sneaker' males seem to mimic females, which suggests not only a condition-dependent alternative mating tactic but potentially rather an alternative mating strategy based on genetic polymorphism (Gross 1996). 'Sneaker' males in common gobies were so far described in the literature as 'small' males of the population (but still clearly recognisable as males, e.g. black anal fin), unable to establish and defend a nest but with a life history not different from the large nest-holding males (but see for Britain: Tipping 1991; see for our study area: Magnhagen 1992; Magnhagen 1994; Magnhagen 1998). Thus, it was assumed till now that the difference in the behaviour of common goby males of different sizes is depending on ontogenetic development, changing with growth. Males not displaying any male phenotypic traits (FMs) at all, however, might be the result of genetic polymorphism (Lank et al. 2013). Further investigation is needed to assess if the female mimics phenotype is genetically different to other males or sequential like in the peacock blenny (*Salaria pavo*; Goncalves et al. 2005). We only found males mimicking females in high and intermediate salinity sites. A possible explanation might be the overall lower population density at low salinity sites (see Chapter I), reducing male-male competition over access to females and making the occurrence of female mimics redundant.

Our results on testes mass of FMs mirror those in sand gobies (*P. minutus*), where small males without breeding colouration (but still recognisable as males) showed extremely large testes compared to males in breeding colouration (Kvarnemo et al. 2010). It has been shown in fish that with increasing risk and intensity of sperm competition testes size increases and correlated to this ejaculate volume (Parker et al. 1996; Parker et al. 1997; Stockley et al. 1997; Parker and Ball 2005). Female mimics most likely always face strong sperm competition, and thus need to invest in large testes (Parker 1990). To directly assess the intensity of sperm competition, however, it is necessary to know the frequency of NH males versus FM males within populations. Thus, we encourage further research directly addressing the occurrence and abundance of FMs in populations along the salinity gradient in the Baltic Sea.

SDGs of female mimics were significantly smaller than those of other males (RPS, NH), which again coincides with results found for 'parasitic' males of the sand goby (Miller 1984; Kvarnemo et al. 2010). Investing in large testes implies lower investment in SDGs (Immler et al. 2004), which our results support by SDGs making up the lowest proportion of the total gonad mass in FMs compared to all other males. SDGs evolved in males of all Gobiidae (Eggert 1931; Miller 1984; Marconato et al. 1996; Ota et al. 1996; Rasotto and Mazzoldi 2002; Svensson and Kvarnemo 2005) independent of the mating tactic (Fishelson 1991). Morphological patterns of SDGs, however, can reflect adaptations to different mating tactics: in two different goby species (*Z. ophiocephalus* and *G. niger*) small males (more likely to sneak fertilizations) had SDGs containing very little mucus, mainly used for sperm storage, while large males' SDGs produced large amounts of mucus (Rasotto and Mazzoldi 2002; Mazzoldi et al. 2005). From an evolutionary point of view it is obvious why FMs do not heavily invest in SDGs but rather in testes: FMs do not provide a nest for females to spawn in, thus preparing the nest with protective mucus is not necessary, however, withstanding high sperm

competition is. The clear allocation differences in gonadal investment (large testes, small SDGs) in female mimics highlight that we here not just coincidentally discovered odd morphs of males looking like females but that we actually deal with males taking on the role of 'sneaking'.

The relationship of gonad size and salinity

Phenotypically recognisable males (=RPS+NH males of model 1, hereafter referred to as 'males') and nest holding (NH) males of low salinity habitats had significantly larger SDGs than males of high and intermediate salinity sites - both in absolute and relative terms. For males of low salinity sites in general and for NH males (independent of salinity) in particular, large SDGs should be advantageous, because SDG size is assumed to be positively correlated with the production of mucins. Mucins are used to make sperm-containing mucus trails that cover the nest (Kvarnemo et al. 2010) and which can improve sperm viability (Eggert 1931; Fishelson 1991; Miller 1992) and egg survival (Giacomello et al. 2006). In particular, the mucus may protect against egg infection by the fungus *Saprolegnia* in low salinity habitats (Lehtonen and Kvarnemo 2015a; Lehtonen and Kvarnemo 2015b). Compatible with results on SDGs, we found that also the proportion of SDGs of the total gonad mass was largest in males and NH males from low salinity sites. In males where SDGs take up the larger proportion of the total gonad mass (in our study on average 60 % for males originating from low salinity sites) a trade-off between investing in SDGs or investing in testes is expected (Immler et al. 2004). What weakens the idea of such a gonadal investment trade-off, however, is the absence of differences in testes mass of males originating from different salinity levels. Our prediction for NH males of intermediate salinity sites to show larger testes due to a higher population density (see Chapter I) increasing the intensity of sperm competition (Brown and Brown 2003) was

not verified. Such allocation differences in gonadal investment highlight the importance to investigate testes and SDGs in Gobiidae separately to avoid false conclusions about the investment into gonads.

The relationship of liver size and salinity

Liver size, which can fluctuate over time as a function of social status, migration, feeding and reproduction (Allen and Wootton 1982; Piersma and Lindstrom 1997; Sopinka et al. 2009) was significantly larger in males and NH males as well as in females of low salinity sites than of intermediate or high salinity sites. The consistency in the results for both sexes of low salinity sites, support the assumption that geographical variation in salinity or its potential ecological consequences provides insights into adaptive evolution, in this case by enlarged energy reserves (see also Chapter II). A large liver may be a prerequisite to invest heavily in large gonads necessary in low salinity habitats (see above), which is supported by a strong relationship between gonad mass and liver mass (Person correlation coefficient, $\rho = 0.60$). Large livers could also indicate an increased energy intake (Allen and Wootton 1982), however, low salinity sites in the Baltic Sea rather represent poor quality habitats for many fish species (see also Chapter I; Möllmann and Koster 2002; Möllmann et al. 2005; Vuorinen et al. 2015). Thus, greater access to food or an overall better body condition of fish is therefore rather unlikely.

Testing variation among all three male categories without accounting for salinity levels (model 3) there was no pairwise difference in liver mass among males. Nevertheless, male category seemed to have an effect (see Table 8) but the low sample size of female mimics and the strong effect of salinity might prevent the detection of significant differences. This underlines that salinity itself has a strong effect on the amount of stored energy reserves in *P. microps*

and that variation of salinity may mask other more subtle variation of liver mass in relation to male reproductive roles. The lack of a significant differentiation in liver mass between male categories may suggest selection by salinity on the level of energy reserves necessary in common gobies.

Here, we assume that liver size is related to spawner quality and/or energy reserves stored (Marshall et al. 1999). However, we did not measure liver glycogen, lipids and protein levels, nor have they been related to size/mass differences in other studies. Thus, liver mass may not be a sufficient energy metric. Nevertheless, consistent differences in liver mass between salinity levels warrant further investigations into the histology measuring liver glycogen, lipids and protein levels of the liver in populations living along the salinity gradient in the Baltic Sea, with a special focus on individuals of low salinity habitats.

Conclusion

There is no doubt that the direct environment in which an organism lives is influencing different aspects of its life history, and therefore growth, reproduction and survival. With this study we contribute empirical evidence that abiotic factors can influence gonadal investment and even more so the storage of energy reserves in marine fish. Providing here the basis of differences in gonadal investment between males originating from different salinity habitats, we suggest to further investigate which male type uses which suite of ARTs in the field along the salinity gradient in the Baltic Sea. This would allow to link environmental heterogeneity to the fitness of ARTs. A sufficient explanation for livers to be larger at low salinity sites is lacking. Thus, and because liver size might not reflect energy stores sufficiently well, we suggest to examine liver glycogen, lipid and protein levels. The most surprising finding of this study was, however, the existence of female mimics. Thus, for future studies investigating gonadal

investment and ARTs we encourage to include females to avoid overlooking the real frequency of 'sneakers' within a population.

Acknowledgements

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Summary and discussion

A major goal of evolutionary biology is to understand the origin of phenotypic and genetic population divergence (Wright 1931). Studying adaptation and evolution at geographically and temporally large scales is hereby a major problem. A solution to this dilemma is to investigate the distribution of organisms and their genetic and phenotypic variation as a function of environmental tolerances and adaptation across environmental gradients, where abiotic and biotic parameters gradually change in space and time (Doyle et al. 2010; Jennings et al. 2013). Analysing ecological variation across a salinity gradient in the Baltic Sea I could show that an abiotic factor like salinity may lead to phenotypic and genetic population divergence.

I first aimed to fill the lack of knowledge about nest resource quantity and quality along the complete salinity gradient in the Baltic Sea, including intermediate salinity sites to assess geographic variation of an abiotic factor fundamental for mating success in the mussel-breeding common goby (*Pomatoschistus microps*, Chapter I). I counted *Mya arenaria* mussel nests and assessed their size distribution, and found a clear decrease in quantity and quality (size) from West (high salinity) to East (low salinity). The assessment of nesting resources, being the most important prerequisite for successful mating in many fish species with a resource-based mating system is crucial, to be able to fully evaluate processes and outcomes of sexual selection (Forsgren et al. 1996b), natural selection and population divergence (Schluter 2000). Shortage or poor quality of nesting resources may affect sexual selection, i.e. male-male competition as well as female choice (Lindström 1992; Borg et al. 2002; Takahashi and Kohda 2002; Lehtonen et al. 2007). That sexual selection and natural selection are strongly

linked is known from examples such as the '*sensory drive hypothesis*', where both male mating traits as well as the perceptual system that underlies female preferences adapt to local environments (but see for other examples on the interaction of natural and sexual selection e.g. Endler 1983; Boughman 2002; Seehausen et al. 2008; Safran et al. 2013). However, the relative strength of divergent selection (natural and sexual selection) in heterogeneous environments is in many species still poorly understood (but see Svensson et al. 2006; Labonne and Hendry 2010; Morgans et al. 2014). To evaluate variation in mating- and reproductive success I further conducted a standardized mating assay along the salinity gradient. I found the highest mating success at low salinity sites (i.e., sites with a low natural nest resource availability). However, mating success was not positively correlated with reproductive success (i.e., brood size), which was highest at the intermediary site offering the highest number of natural nests. Since the fitness currency of sexual selection can be viewed as either number of mates (i.e., mating success) or number of offspring (i.e., reproductive success; Wade 1979; Wade and Arnold 1980; Arnold and Wade 1984) it is not feasible to make any clear statements about differences in the strength of sexual selection between the investigated populations at this point. However, I could highlight that ecological context may lead to variation in sexual selection between populations and suggest further field experiments testing variation in mating- and reproductive success between populations along the Baltic salinity gradient. Furthermore, along with nest quantity and quality it was body size of *P. microps* that showed the strongest co-variation with salinity. Body size of Baltic common gobies showed a clear decrease with decreasing salinity from West to East and male body size was highly correlated with *M. arenaria* nest size.

After pointing out in Chapter (I) how fundamental aspects of mating success like resource availability and body size can co-vary with an abiotic factor like salinity, I aimed to investigate

in my second study the relative role of neutral and adaptive evolution in driving population divergence and adaptation to salinity in common gobies (Chapter II). Along with the results on body size (Chapter I) as well as gonadal- and liver investment (Chapter III) showing a clear difference between western and eastern Baltic populations I found low salinity populations (East) to form a monophyletic group within the Baltic cluster (Chapter II). Even though at this point I cannot exclude phenotypic plasticity being the cause for smaller body size and larger gonads (i.e., SDGs) and livers in individuals of low salinity sites, eastern Baltic populations forming a monophyletic group may suggest divergent selection acting, resulting in different phenotypes in different (salinity) environments. This assumption is also supported by the results of the P_{ST} - F_{ST} comparison, which revealed that body shape (often closely linked to variation in body size; Araujo et al. 2014) of Baltic common gobies showed to be under divergent selection (Chapter II). Eastern Baltic populations being locally adapted to low salinity may cause reduced gene flow between high and low salinity sites and thus drive population divergence (see also DeFaveri and Merila 2014). I expected GDM results to support the assumption of isolation by adaptation for low salinity populations resulting in a pattern of IBE (likely to be driven by salinity) explaining genetic differentiation between western and eastern Baltic populations; however, neither IBD nor IBE explained population divergence in Baltic common gobies. A possible explanation could be the scenario of 'isolation by colonisation', which is neutral and thus no assumption about non-neutral genetic variation in relation to space or environment can be made (Orsini et al. 2013; Spurgin et al. 2014; DeWoody et al. 2015). My results add to a slowly but steadily growing pool of literature on species inhabiting the Baltic Sea showing genetic and phenotypic dissimilarity on a fine geographic scale between western and eastern Baltic populations as a result of divergent selection (e.g. Olsson et al. 2012; Teacher et al. 2013).

In my third study (Chapter III) investigating gonadal and liver investment in Baltic common gobies I found again differences between high and low salinity populations. Males had larger sperm duct glands (SDGs) and males as well as females had larger livers at low salinity sites; according to results of Chapter II are western and eastern Baltic populations genetically distinct and thus, such differences in gonadal- and liver investment may have a genetic basis. It is likely that different genotypes with differences in expression of gonadal- and liver investment reaction norms are favoured at different sites along the gradient, leading to population divergence. The different allocation in gonads between West and East as well as males of low salinity investing more in SDGs than testes does coincide with the investigated genetic structure (Chapter II) but also with ecological differences caused by salinity (Chapter I). Larger mucus producing SDGs may be needed to protect eggs, which bear a higher risk of egg mold infections in low salinity (Lehtonen and Kvarnemo 2015a). Also larger livers in low salinity may be needed to store enough energy necessary for osmoregulation (Tseng and Hwang 2008). Thus, a very likely explanation for differences in gonadal-and liver investment between high and low salinity populations is isolation by adaptation to salinity resulting in a pattern of IBE promoting population divergence (Chapter II).

During dissection I detected for the first time in this species 'female mimics', i.e. fish that were categorised by visual inspection as females but had male gonads. Such males mimicking females, however, were only detected at high and intermediate but not at low salinity sites. At low salinity sites I found low nest availability (Chapter I), thus being opportunistic and adopt a bourgeois tactic if the opportunity of monopolising a nest comes up, seems advantageous. This is an option for ontogenetic sneaker males in common gobies (as they used to be described in the literature, see Magnhagen 1992). However, males mimicking females as I found them in high and intermediate salinity sites, do not have the option of adopting the

bourgeois tactic. Alternative tactics evolve when there is fitness to be gained by divergent allocation tactics (Oliveira et al. 2008), thus mimicking females in the high energy demanding low salinity environment (as results of Chapter I: small body size and low population density and Chapter III: high SDG- and liver investment, indicate) might not be an evolutionary stable strategy (Tomkins and Hazel 2007). Female mimics had significantly larger testes but smaller SDGs than all other males, a pattern similar to that found in 'sneaker' males in the closely related sand goby (Kvarnemo et al. 2010). Therefore, I am certain to have not coincidentally discovered odd male morphs but actual 'sneakers' that possibly even resulting from genetic polymorphism (i.e. true alternative tactics; Shuster and Wade 1991; Gross 1996; Tuttle 2003).

Synopsis

In the course of this thesis, I aimed to understand how variation in ecology along an environmental gradient can influence fundamental aspects of the mating success in the common goby (*Pomatoschistus microps*), possibly driving population divergence.

My first study highlights the importance to consider ecological factors promoting a basis for environmental-dependent divergent selection (natural and sexual selection). I provide valuable background information for further studies on population divergence and adaptation to salinity in general.

In my second study I wanted to investigate if Baltic common gobies show a distinct genetic structure and possibly local adaptation to salinity. My conclusion of the first study was supported by the outcomes of my second study, where I showed that an environmental factor such as salinity can drive divergent natural selection, leading to a clear genetic structure as well as phenotypic differentiation. Even though fully disentangling neutral (IBD) and adaptive (IBE) evolutionary processes driving population divergence was difficult because of spatial variation, I could nevertheless show that it is often not only IBD but also IBE driving population differentiation.

In my third study I found further evidence supporting the assumption of salinity promoting population divergence, with males of low salinity showing larger gonads (i.e., sperm duct glands) and males as well as females larger livers.

Synopsis

Overall I conclude that common goby populations from low salinity sites in the Baltic Sea show a clear morphological and genetic distinction highlighting that ecology can drive adaptive population divergence and limit gene flow as the first steps towards ecological speciation.

Outlook

Chapter I:

I suggest further research on sexual size dimorphism (SSD; see Fig. 21) in common gobies along the salinity gradient in the Baltic Sea. My results showed that females generally tend to be slightly larger than males, especially early in the season (Fig. 6; reversed Rensch's rule). However, since females of all population grew and were significantly larger late than early in the season, while males grew only in high and intermediate salinity populations, there is a shift in SSD over the season as well as a difference in the SSD between populations along the salinity gradient. SSD can mirror the relative importance of natural and sexual selection on both sexes (Shine 1989) and effects of spatial as well as temporal variation in SSD within and between populations can be investigated along the salinity gradient in the Baltic Sea.

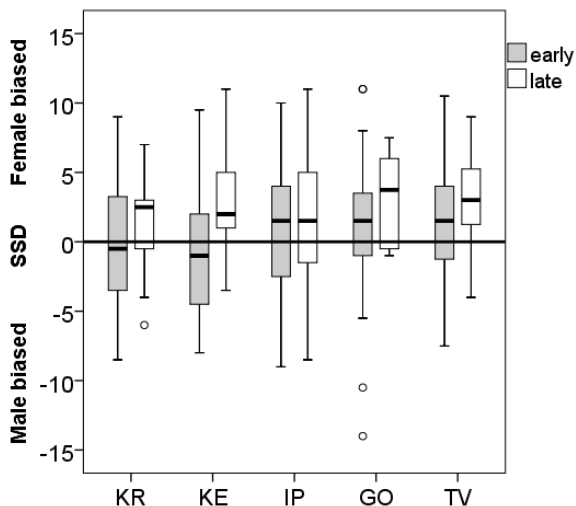


Figure 21. Sexual size dimorphism (SSD = centred female length - centred male length) of common gobies calculated from body size data collected over three consecutive years early (grey boxes) and late (empty boxes) during the breeding season in the Baltic Sea along the salinity gradient at five populations. Negative values indicate a male biased SSD (i.e. Rensch's rule, males are larger than females) and positive values indicate a female biased SSD (i.e. reversed Rensch's rule, females are larger than males).

Chapter II:

Supported by GDM results showing IBE to drive population divergence as well as by the P_{ST} - F_{ST} comparison suggesting phenotypic population differences due to divergent selection, I suggest common gobies to be genetically adapted to salinity. However, the only way to really prove genetic adaptation and to exclude phenotypic plasticity is a full-factorial common garden experiment. With such an experimental set up, one could at the same time also assess if gonadal and liver mass differences show a genetic adaptation to salinity or are caused by phenotypic plasticity (Chapter III).

Chapter III:

Salinity had a clear effect on gonadal investment (i.e., sperm duct glands). Also male tactic (bourgeois males versus female mimics) seemed to affect gonadal investment, however, sample size of female mimics was very low and thus no connection to salinity could be made regarding gonadal investment in female mimics. Thus, I encourage further research on how salinity affects gonadal investment in males following different tactics, especially including a higher sample size of female mimics. Only then it is possible to disentangle environmental-related gonadal investment (e.g. salinity related) and tactic-related gonadal investment to not draw wrong conclusion about the fitness of alternative reproductive tactics. Furthermore, it is necessary to investigate if differences in gonadal and liver investment are caused by phenotypic plasticity or due to divergent selection (see above 'common garden experiments').

List of contributors and affiliations

Isabel M. Mück^a conducted and developed all experimental ideas and tests in the field, authored this dissertation and the resulting manuscripts, conducted all population genetic procedures in the laboratory, geometric morphometric measurements and all data analyses.

Katja U. Heubel^{ab} supervised Chapter I and Chapter III, gave advice and helped with experimental set ups, data analyses and the overall aim of manuscripts and this dissertation, assisted partly at field work.

Kenyon B. Mobley^c and **Henri A. Thomassen**^a supervised Chapter II, gave advice on population genetic laboratory procedures and analysis, contributed critically to Chapter II.

Phillip Gienapp^d contributed support in adjusting a R script after Whitlock and Guillaume (2009) for the P_{ST} - F_{ST} comparison and contributed critically to Chapter II.

Judith Stauss^a dissected all fish and weight gonads and livers for analyses in Chapter III.

^a University of Tübingen, Department of Biology, Institute for Animal Evolutionary Ecology, Auf der Morgenstelle 28, 72076 Tübingen, Germany

^b Present address: Institute for Zoology, Grietherbusch Ecological Research Station, University of Cologne, Germany

^c Max Planck Institute for Evolutionary Biology, Department of Evolutionary Ecology, August-Thienemann Str. 2, 24306 Plön Germany

^d Department of Animal Ecology, Netherlands Institute of Ecology, 6700 AB Wageningen, The Netherlands

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