Effekte von Xenohormonen auf die limnischen Invertebraten *Gammarus fossarum* (Crustacea, Amphipoda) und *Marisa cornuarietis* (Mollusca, Prosobranchia)

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1. Promotionsthema

Effekte von Xenohormonen auf die limnischen Invertebraten *Gammarus fossarum* (Crustacea, Amphipoda) und *Marisa cornuarietis* (Mollusca, Prosobranchia)

2. Einleitung

Seit den frühen 50er Jahren des letzten Jahrhunderts deuten Untersuchungen immer wieder darauf hin, dass in der Umwelt Substanzen existieren, die mit dem Hormonsystem von Organismen interagieren können, wodurch nachteilige Auswirkungen für die betroffenen Tiere entstehen (Colburn, 2002; Guillette & Gunderson, 2001; McLachlen, 2001). Vor ungefähr 15 Jahren wurde daraufhin der Begriff „Endokrine Disruption" eingeführt, der diese Vorgänge beschreiben soll. Trotz der langen Zeit seit Bekanntwerden des Phänomens und vielen neuen Erkenntnissen auf diesem Gebiet, ist eine allgemein gültige Definition dieses Begriffes auch heute noch nicht gegeben. Zu den sogenannten *`endocrine disruptors´* (EDs) oder *`endocrine disrupting chemicals´* (EDCs), auch `Umwelthormone´ genannt, gehören Substanzen und deren Metabolite, die mit der Produktion, der Freisetzung, dem Transport und dem Abbau körpereigener Hormone interagieren, sowie mit der Wirkung von Hormonen auf deren Rezeptor konkurrieren. Es handelt sich dabei um Umweltchemikalien, die in ansonsten nicht toxischer Konzentration in das hormonelle System von Mensch und Tier eingreifen können. Eine beispielhafte Definition aus dem Jahre 1997 für diese Stoffe bietet die US-Umweltbehörde (EPA): „*An environmental endocrine disrupter is defined as an exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostatis, reproduction, development, and/or behavior*". Zu diesen Substanzen zählen zum einen natürlich vorkommende Hormone wie z.B. Estrogene und Androgene, Phytohormone, sowie künstlich hergestellte Hormone wie sie unter anderem in Kontrazeptiva verwendet werden. Darüber hinaus zählen aber auch Chemikalien zu den EDCs, die auf Grund ihrer strukturellen und chemischen Eigenschaften hormonell wirksam sein können. Der Begriff Xenohormone umfasst alle diese Substanzen, wobei sich das Präfix „Xeno" (Fremd) darauf bezieht, dass sie nicht vom eigenen Organismus gebildet wurden.

Die Einteilung der Chemikalien erfolgt nicht nach ihrer Herkunft, sondern nach ihrer

Wirkung und kann hinsichtlich der Wechselwirkung mit Sexualhormonen wie folgt in vier Gruppen vorgenommen werden: Verbindungen mit estrogenen Wirkungen, Verbindungen mit anti-estrogenen Wirkungen, sowie Verbindungen mit androgenen bzw. anti-androgenen Wirkungen. Diese Einteilung basiert ausschließlich auf der Fähigkeit der Substanzen zur Interaktion mit natürlichen Sexual-Steroidhormonen und wird daher nicht allen Substanzen und Effekten gerecht. Zudem ist es möglich, dass ein und derselbe Stoff auf unterschiedliche Weise in das Hormonsystem eingreifen kann und somit z.B. aktivierend und inhibitorisch zugleich wirken kann. Die weitaus größte und am besten untersuchte Gruppe ist die der estrogen wirkenden Substanzen. Hierzu gehören neben den endogenen Steroidhormonen wie Estradiol und Estron auch die exogenen Steroidhormone, welche sich fast ausschließlich von 17β-Estradiol ableiten. Diese synthetischen Hormone, wie z.B. 17α-Ethinylestradiol oder Mestranol, werden von der pharmazeutischen Industrie in großen Mengen hergestellt und haben ihren Einsatz vor allem in der hormonellen Empfängnisverhütung sowie in therapeutischen Zwecken. Darüber hinaus besitzen eine Vielzahl von Xenohormonen ein estrogenes Potential, welches nicht beabsichtigt ist. Dazu gehört unter anderem das 4-Nonylphenol aus der Gruppe der Alkylphenole, das in Europa immer noch in großen Mengen produziert wird (1995 ~ 64000 t; Fent, 1998), aber auch die polychlorierten Biphenyle (PCB), deren schädliche Wirkungen bereits seit langem bekannt sind (Soto et al., 1995; Bitman & Cecil, 1970). Darüber hinaus sind Verbindungen wie DDT und dessen Derivate sowie Vertreter der polyzyklischen aromatischen Kohlenwasserstoffe (PAK) oder der Bisphenole zu erwähnen. Auf letztere wird am Beispiel von Bisphenol A (BPA) in Kapitel 3 und Kapitel 6 noch genauer eingegangen. BPA, das in großen Mengen produziert wird, steht seit geraumer Zeit im Verdacht, estrogene Wirkung zu haben, was auch bereits in mehreren Studien nachgewiesen werden konnte (Stocker et al., 2003; Jobling et al., 2002; Schönfelder et al., 2002; Staples et al., 1998).

Anti-estrogene Effekte sind bei weitem nicht so gut untersucht und deshalb lassen sich auch nur einige Verbindungen mit solcher Wirkung nennen. Aus der Brustkrebstherapie ist z.B. das Tamoxifen bekannt, welches kompetitiv an den Estrogenrezeptor bindet, aber auch aus der Gruppe der PCB und der PAK sind vor allem koplanare Substanzen mit anti-estrogenen Wirkungen bekannt, die den Ah-Rezeptor blockieren (Poland & Knutson, 1982). Die einzigen nicht-steroidale Verbindungen in der Umwelt, denen eine androgene Wirkung zugeschrieben werden kann, sind derzeit Organozinn-Verbindungen, z.B. Tributylzinn (TBT), das verantwortlich für das Imposexphänomen bei prosobranchen Schnecken ist und somit die

Fortpflanzungsfähigkeit weiblicher Schnecken beeinträchtigt (Bettin et al., 1996; Oehlmann et al., 1996; Gibbs & Bryan, 1986). Neben dem Einsatz von Anti-androgenen für therapeutische Zwecke ist eine derartige Wirkung auch bei einigen Pestiziden wie z.B. bei Linuron und Diuron bekannt (Cook et al., 1993).

Die beschriebenen Substanzen werden zum Teil in sehr großen Mengen produziert und gelangen über Kläranlagen, die Landwirtschaft, Sickerwasser von Deponien oder die Luft bzw. den Niederschlag in unsere Gewässer. Auch wenn diese Chemikalien längst nicht mehr im Einsatz sind, so lassen sich einige von diesen auf Grund ihrer hohen Persistenz noch Jahre später in der Umwelt, vor allem in Organismen am Ende der Nahrungskette nachweisen. Ein bedeutendes Beispiel dafür ist sicher das Insektizid DDT, das mit am Anfang der Erforschung endokrin wirkender Chemikalien stand. In den letzten 50 Jahren wurde eine Vielzahl von Untersuchungen an frei lebenden Populationen durchgeführt, wie z.B. an Vögeln, Alligatoren und Säugern (Lundholm, 1997; Semenza et al., 1997; Guillette et al., 1996; Mason et al., 1986; Spitzer, 1978); zudem wurde in jüngster Zeit vermehrt angestrebt, den Mechanismus, vieler dieser beobachteten Effekte durch Laborversuche besser zu verstehen. Obwohl mindestens 95% der bekannten Arten Invertebraten sind, liegen bislang leider nur sehr wenige Studien vor, die sich mit den Auswirkungen von EDCs auf diese Organismen beschäftigen (de Fur et al., 1999). Ein Grund hierfür ist das oft noch lückenhafte Wissen über die hormonellen Vorgänge und Mechanismen bei vielen Invertebraten und die großen Unterschiede, sowohl zwischen als auch innerhalb der verschiedenen Phyla.

Generell scheint es so zu sein, dass sich Unterschiede vor allem zwischen Protostomiern und Deuterostomiern bezüglich der auftretenden Hormone feststellen lassen, mehr als zwischen Vertebraten und Invertebraten. Begründet wird dies vor allem mit dem Auftreten von für Vertebraten typischen Sexual-Steroiden wie Progesteron und Testosteron bei Echinodermen (Voogt et al., 1991). Bei Invertebraten kommen im Allgemeinen drei Typen von Hormonen vor: Steroide, Terpenoide und Peptidhormone, wobei die Peptidhormone bei weitem am häufigsten sind. Steroide werden bei Invertebraten in der Regel in Drüsen neuronalen Ursprungs gebildet und diese werden daher auch als neurosekretorische Gewebe bezeichnet. Bei den Crustaceen, die in dieser Arbeit anhand des Flohkrebses *Gammarus fossarum* untersucht wurden, ist eines der wichtigsten Steroidhormone das Ecdyson. Wie auch bei den Insekten ist es vor allem für die Häutung der Tiere verantwortlich und wird durch das *molt inhibiting hormone* (MIH), das im X-Organ des Augenstiels gebildet wird, kontrolliert. Vom Mandibularorgan wird das Hormon Methylfarnesoat gebildet, das mit dem

Juvenilhormon der Insekten verglichen werden kann und maßgeblich an stimulatorischen Effekten während der Reproduktion beteiligt ist. Bei Amphipoden und anderen Crustaceen wird die Ausbildung der primären und sekundären Geschlechtsmerkmale vom Androgenen Hormon gesteuert. Die positive regulatorische Kontrolle dieses Hormons konnte durch Implantatversuche der Androgenen Drüse, die sich bei Gammariden am *Vas deferens* befindet, nachgewiesen werden (Nagamine et al., 1980). Fehlt das Androgene Hormon, hat dies zur Folge, dass sich funktionelle Weibchen entwickeln.

Als zweiter Versuchsorganismus wurde in dieser Arbeit die Apfelschnecke *Marisa cornuarietis* untersucht. Sie stellt einen Vertreter der Gastropoden dar, bei denen, wie auch bei allen anderen Mollusken, typische Steroide anderer Invertebraten wie z.B. Ecdyson oder das Juvenilhormon selten vorkommen. Es wird aber angenommen, dass Gastropoden in der Lage sind, bei Vertebraten auftretende Steroide wie Progesteron oder Testosteron zu synthetisieren oder zumindest zu verschiedenen Derivaten metabolisieren zu können (Oberdörster et al., 1998; Wootton et al., 1995). Ein bedeutendes Neuropeptidhormon ist bei Schnecken das *egg-laying hormone* (ELH) das verantwortlich für die Reifung, die Eiproduktion und das Legeverhalten der weiblichen Tiere ist.

Am Beispiel von *M. cornuarietis* kann, wie bereits erwähnt, deutlich gezeigt werden, auf welche Weise endokrine Disruptoren Einfluss auf das Hormonsystem haben können. Das Pestizid Tributylzinn (TBT) ist verantwortlich für das Imposex-Phänomen, das durch die Bildung eines Penis und eines V*as deferens* bei weiblichen Schnecken charakterisiert ist. Es wird angenommen, dass TBT die Cytochrom-P_{450}-abhängige Monooxygenase (= Aromatase) hemmt und somit die Umwandlung von Testosteron zu 17β-Estradiol verhindert (Morciollo et al., 1998). Der ansteigende Testosteronspiegel führt daraufhin zur Ausbildung männlicher Geschlechtsmerkmale. Es ist selten, dass der Zusammenhang zwischen einer Substanz und ihrer Wirkung so eindeutig hergestellt werden kann; vor allem bei Wirbellosen, über die bislang nur wenige Studien vorliegen. Sogenannte Intersex-Tiere, die sowohl männliche als auch weibliche Geschlechtsmerkmale aufweisen, sind bei Crustaceen bereits bekannt und für viele Arten beschrieben. So wurde in Kapitel 4 dieser Arbeit untersucht, ob das Intersex-Phänomen auch bei *Gammarus fossarum* natürlicherweise auftritt.

Das Hauptaugenmerk bei EDCs liegt allerdings auf der rezeptorvermittelten Wirkung, worüber in den letzten Jahrzehnten eine Fülle an neuen Informationen gewonnen werden konnte. Durch den erhöhten Anteil weiblicher Tiere in Möwenpopulationen sowie Ovar-ähnliches Gewebe bei männlichen Alligatoren (Guillette et al., 1994; Fry et al., 1987; Fry &

Toon, 1981) wurde man in den 1970er Jahren auf die unerwünschten Effekte von DDT und dessen Metabolite aufmerksam. Bei denselben Untersuchungen wurden unter anderem verkümmerte Reproduktionsorgane bei Männchen festgestellt, was sich ebenfalls auf das Insektizid DDT zurückführen ließ (Guillette et al., 1996). Neben DDT sind es aber auch andere Pestizide, synthetische Estrogene, Alkylphenole, wie 4-Nonylphenol oder Bisphenole, wie BPA, die ein endokrines Potential besitzen. Allen gemeinsam ist, dass ihre Wirksamkeit darin begründet liegt, dass sie an intrazelluläre Estrogenrezeptoren (ER) binden. Dieser Komplex aus Rezeptor und Hormon / Xenohormon wiederum bindet im Zellkern an *Estrogen Response Elements* (ERE) der DNA, wodurch in Folge verschiedene Gene aktiviert werden können. Hsp90 nimmt bei diesem Vorgang eine wichtige Rolle ein, da es unerlässlich für die Bindung der jeweiligen Substanzen an den Rezeptor ist. Obwohl die Affinität der Xenoestrogene zum Rezeptor meist wesentlich geringer als die der natürlichen Hormone ist, kommt es trotzdem zu einer Bindung und einer Aktivierung des Rezeptors. Ein weiteres Problem ist die hohe Persistenz dieser Stoffe in der Umwelt und ihre Fähigkeit zur Bioakkumulation im Gewebe vieler Organismen.

Entgegen der weit verbreiteten Auffassung, dass ausschließlich bei Deuterostomiern Estrogenrezptoren vorhanden sind, die einen solchen Effekt vermitteln können, mehren sich in den letzten Jahren Hinweise, dass dies wahrscheinlich nicht der Fall ist. Di Cosmo et al. (2002) beschreiben in ihrer Arbeit einen ER bei *Octopus vulgaris* und Thornton et al. (2003) vermuten, dass der ursprüngliche Steroidrezeptor ein Estrogenrezeptor war und daher auch bei den Protostomiern noch in einigen Gruppen erhalten sein könnte. Die Ergebnisse in Kapitel 5 dieser Arbeit weisen ebenfalls in diese Richtung und zeigen ein ERα-ähnliches Protein bei *G. fossarum*. Dies könnte einige der bisher beobachteten Effekte bei Invertebraten erklären, welche durch Estrogene induziert werden könnten (Duft et al., 2003; Pascoe et al., 2002; Kirkebride-Smith et al., 2001; Watts et al., 2001a). Ob diese Rezeptoren in den jeweiligen Organismen allerdings funktionsfähig sind, bleibt noch zu klären. Sollte sich dies aber bewahrheiten, würde den Invertebraten in der Diskussion über die Wirkung von EDCs eine ganz neue Bedeutung zukommen.

Zum heutigen Zeitpunkt stehen eine ganze Reihe an Tests zur Verfügung, um das estrogene Potential von Chemikalien abzuschätzen. Einer dieser Biomarker ist z.B. die Induktion von Vitellogenin bei männlichen Organismen. Der Vorläufer des Dotterproteins wird in der Leber von ausschließlich weiblichen Fischen, Vögeln, Reptilien und Amphibien zur Versorgung der Embryonen gebildet. Vitellogenin wird nur unter der Einwirkung von

Estradiol gebildet, kann aber auch bei Männchen und Jungtieren nachgewiesen werden, wenn diese durch estrogen wirkende Substanzen beeinflusst wurden (Hutchinson & Pickford, 2002; Sumptor & Joblin, 1995). Weitere *In-vivo* Tests auf estrogene Wirkungen erfassen Parameter wie das Uterus- oder Oviduktgewicht, den Glykogengehalt des Uterus oder die Geschlechtsdifferenzierung bei Reptilien, Vögeln, Mäusen und Ratten. Darüber hinaus wird in *In-vitro* Tests z.B. das Verhalten Estrogen-sensitiver Brustkrebszellen oder die Affinität verschiedener Substanzen zum Estrogenrezeptor untersucht. Anders als bei den gängigen ökotoxikologischen Untersuchungsmethoden, bei denen Invertebraten bereits eine wichtige Rolle spielen (Daphnientest; Hammers-Wirtz & Ratte, 2000), gibt es bei der Erfassung von endokrinen Disruptoren mit Ausnahme des Imposex-Effektes bei Prosobranchiern bisher so gut wie noch keine geeigneten Tests mit Invertebraten. Der in Kapitel 6 beschriebene Embryotest mit *Marisa cornuarietis* könnte ein Beitrag sein, um diese Lücke in Zukunft zu schließen. Sogenannte *early life stage* Tests mit *Danio rerio* haben bereits bewiesen, dass sie über eine grosse Aussagekraft verfügen, nicht zuletzt weil sie frühe, sensitive Entwicklungsstadien miteinschließen und auf Grund ihrer kurzen Dauer einen Vorteil gegenüber *life cycle* Tests bieten (Nagel, 2002). Die Tatsache, dass über 95% aller bekannten Arten Invertebraten sind und dass diese die Grundlage für die meisten Nahrungsketten darstellen, sollte die Bemühungen noch verstärken, die Auswirkungen von Chemikalien mit endokrinem Potential auf Invertebraten besser verstehen zu können.

Das Ziel der vorliegenden Untersuchungen war es, festzustellen, (1) inwiefern Änderungen im Gehalt des bei Vertebraten die Steroidwirkung modulierenden Proteins hsp90 sowie des unspezifischen proteotoxischen Streß induzierenden Proteins hsp70 aus der Oocytenreifung bei *G. fossarum* resultieren. Des weiteren sollte auf dieser Grundlage gezeigt werden, (2) ob Abwässer, die mit Xenohormonen belastet sind, einen Einfluss auf im Freiland lebende Gammariden besitzen. Dies sollte sowohl anhand der biochemischen Marker hsp90 und hsp70, als auch auf histologischer Ebene durch die Untersuchung der Oocyten ermittelt werden. In einem nächsten Schritt sollte anhand der gleichen Endpunkte geklärt werden, (3) ob ähnliche Effekte wie im Freiland auch bei Gammariden auftreten, die innerhalb von Mesokosmos-Systemen ("Fließrinnen"), gegenüber verschiedenen Konzentrationen an BPA exponiert wurden. Um eine mögliche mechanistische Erklärung für die beschriebenen Effekte zu liefern, wurde untersucht (4) ob ein Estrogenrezeptor bei *G. fossarum* nachzuweisen ist und dieser sich gegebenenfalls quantitativ durch 17α-Ethinylestradiol induzieren lässt.

Ethinylestradiol induzieren lässt.
Um ein standardisiertes Testverfahren zu ermöglichen, sollte (5) ein Embryotest mit *M. cornuarietis* entwickelt werden, der es ermöglicht, Effekte endokrin wirkender Substanzen anhand von ausgewählten Entwicklungsparametern nachzuweisen.

3. Material und Methoden

3.1 Experimenteller Aufbau

Für die histologischen und biochemischen Untersuchungen während der Eireifung wurden Gammariden aus dem Goldersbach entnommen, ein weitestgehend unbelastetes, kleines Fließgewässer nahe Tübingen, im Südwesten Deutschlands. Die Tiere, die zu Beginn des Experiments alle den gleichen Entwicklungsstand aufwiesen, wurden anschließend in einem Aquarium unter konstanten Bedingungen gehalten, wobei alle 14 Tage Proben für die jeweiligen Untersuchungen entnommen wurden (Kapitel 1).

Um Einflüsse von Klärwerksabwässern mit estrogenem Potential auf die Oocytenentwicklung und die Level von hsp90 und hsp70 zu erfassen (Kapitel 2), wurden an zwei unterschiedlichen Bächen, jeweils vor und nach einem Einleiter, Gammariden entnommen. Die Körsch bei Stuttgart fließt durch den hoch industrialisierten Mittleren Neckarraum und weist eine komplexe Belastung auf (Adam et al., 2001; Honnen et al., 2001), der Lockwitzbach befindet sich in der Nähe von Dresden und wird besonders durch eingeleitete kommunale Abwässer belastet. Die entnommenen Tiere wurden jeweils für die Histologie und die biochemischen Untersuchungen vor Ort fixiert und für die weiteren Arbeitsschritte ins Labor gebracht. Die Gammariden, die hinsichtlich des Auftretens von Intersexen untersucht wurden (Kapitel 4), stammten ebenfalls von den gleichen Probestellen an Körsch und Lockwitzbach.

Für das in Kapitel 3 beschriebene Experiment wurden 5 künstliche Fließrinnen des Instituts für Hydrobiologie der TU Dresden verwendet. Die jeweils gleich aufgebauten Rinnen wurden mit Sediment und Kunstwasser gefüllt und eine definierte Anzahl an Gammariden aus einem unbelasteten Bach bei Dresden (Zschonerbach) vor Beginn der Exposition eingesetzt. Die Tiere wurden maximal 103 Tage lang gegenüber drei Konzentrationen an Bisphenol A exponiert. Proben für die Histologie sowie für die biochemischen Untersuchungen wurden vor Beginn der Exposition, am Ende und zu zwei weiteren Zeitpunkten genommen.

Praecopula-Pärchen von *Gammarus fossarum* wurden aus dem Quellbereich der Steinlach entnommen und im Labor, nach Geschlechtern getrennt, auf das Vorhandensein eines

Estrogenrezeptors untersucht (Kapitel 5). Zudem wurden die Tiere für 5 Tage gegenüber 17α-Ethinylestradiol exponiert, um festzustellen, ob der Level eines solchen Rezeptorproteins responsiv gegenüber Estrogen ist.

Um Auswirkungen von Xenohormonen auf die Embryonalentwicklung von *M. cornuarietis* zu untersuchen (Kapitel 6), wurden Schneckeneier bis zu ihrem Schlupf gegenüber 17α-Ethinylestradiol und Bisphenol A exponiert. Während dieser Zeit wurden für die Entwicklung relevante Endpunkte, wie z.B. das Auftreten der Augen protokolliert und ausgewertet. Um solche Endpunkte in der Entwicklung zu definieren, die eine Reaktion auf die Behandlung mit einer allgemein toxischen Substanz zeigen, wurden die Embryonen im ersten Teil der Methodenentwicklung mit Cadmium behandelt.

3.2 Untersuchte Organismen

Gammarus fossarum KOCH, 1835 ist ein weit verbreiteter Makroinvertebrat in kleinen bis mittelgroßen Fließgewässern Mitteleuropas. Auf Grund seiner Lebensweise ist der Süßwasseramphipode einer der wichtigsten Zerkleinerer in diesen Gewässern und ernährt sich von Pflanzenmaterial, Aufwuchs und Detritus (Engelharder, 1986; Meijering, 1972). In Gewässern, in denen für die Tiere optimale Bedingungen herrschen, können diese in sehr großer Zahl auftreten und nehmen daher auch eine bedeutende Rolle als Nährorganismen für viele Fischarten ein. Nach 3-4 Monaten und etwa 10 Häutungen erreichen die Tiere ihre Geschlechtsreife und messen zu diesem Zeitpunkt ungefähr 6 mm in der Länge (Pöckl & Humpesch, 1990). Von diesem Zeitpunkt an befinden sich die Weibchen ständig in einer Brutphase, die im Sommer 2-3 Wochen lang dauert; bei niedrigeren Temperaturen jedoch wesentlich länger. Davon ausgenommen ist eine Reproduktionspause zwischen Oktober und November. Die entwickelten Eier werden ins Marsupium abgelegt, befruchtet und verbleiben dort bis zum Schlupf der Jungtiere (Kaestner, 1970).

Marisa cornuarietis gehört zu den Ampullariidae und ist in Flüssen und Brackwassergebieten Süd- und Mittelamerikas beheimatet. Im Unterschied zu ihren Verwandten aus der Familie der Ampullariidae besitzt *M. cornuarietis* zusätzlich zu ihren Kiemen eine Lunge. Eine weitere Besonderheit, die sie für die beschriebenen Untersuchungen so interessant macht, ist die Tatsache, dass sie ihr Gelege im Wasser ablegt und nicht außerhalb, wie das bei anderen Apfelschnecken der Fall ist. Die Embryonen entwickeln sich dann innerhalb von 10-14 Tagen in transparenten Eiern, die von einer gallertartigen Masse zusammengehalten werden (Demian & Yousif, 1973).

3.3 Histologische Untersuchungen

Direkt im Anschluss an die Probenahmen wurden die Individuen von *Gammarus fossarum* in Größenklassen eingeteilt ("*large*" (L): ≥ 9 mm in der Länge, "*medium*" (M): ≥ 6 – 9 mm), decapitiert und in 2% Glutardialdehyd (gelöst in 0,005 M Cacodylatpuffer, pH 7,4) fixiert. Vor ihrer Einbettung in Technovit wurden die Tiere in 5% Trichloressigsäure entkalkt, anschließend am Mikrotom Dünnschnitte angefertigt (4 µm Dicke) und diese mit einer Methylenblau-Azur-Lösung (Richardson et al., 1960) zur Übersicht gefärbt. Die Schnitte wurden am Lichtmikroskop untersucht, um die Art der auftretenden Oocyten quantitativ und qualitativ zu erfassen. Die Einteilung der Oocyten erfolge in Anlehnung an Tan Fermin & Pudadera (1989), je nach Reifegrad, in praevitellogene, früh-vitellogene, spät-vitellogene Oocyten, reife Oocyten und bereits wieder im Abbau befindliche Oocyten. Darüber hinaus wurde erfasst, ob die Oocyten intakt oder atretisch waren, um daraufhin bei den intakten Zellen die Fläche mittels digitaler Bildverarbeitung zu ermitteln.

3.4 Biochemische Analysen

Für die biochemischen Analysen wurden die Gammariden direkt nach Entnahme ebenfalls in die gleichen Größenklassen eingeteilt ("*large*" (L): ≥ 9 mm in der Länge, "*medium*" (M): ≥ 6 – 9 mm), in flüssigem Stickstoff gefroren und bis zur weiteren Bearbeitung bei -20°C aufbewahrt. Die Tiere wurden in einem geeigneten Aliquot Extraktionspuffer homogenisiert, anschließend zentrifugiert und der Gesamtproteingehalt des Überstandes nach Bradford (1976) bestimmt. Mit einer nach Laemmli (1970) modifizierten SDS-PAGE wurde die Proteinauftrennung durchgeführt, der nachfolgende Transfer der Proteine aus dem Polyacrylamidgel auf die Nitrocellulosemembran erfolgte mit Hilfe eines Semi-dry Western-Blot. Durch eine Immunreaktion mittels einem ersten und zweiten Antikörper sowie mit anschließender Peroxidasefarbreaktion wurden die Proteine nachgewiesen und die Banden densitometrisch quantifiziert. Für den Nachweis von hsp90, hsp70 und dem Estrogenrezeptor α (ERα) wurden folgende Antikörper verwendet:

Hsp70: 1.AK: *mouse anti-human* hsp70 IgG; 2.AK: *goat anti-mouse* IgG, Peroxidase-Konjugat.

Hsp90: 1.AK: *mouse anti-water mold* hsp90 IgG; 2.AK: *goat anti-mouse* IgG, Peroxidase-Konjugat.

ERα: 1.AK: *rabbit anti-mouse* ERα IgG ; 2.AK: *goat anti-rabbit* IgG (H+L), Peroxidase-Konjugat.

3.5 Embryotest

Die adulten *M. cornuarietis* wurden in Glasaquarien gehalten und ihr Laich täglich von den Wänden abgesammelt. Für den Embryotest wurden die einzelnen Gelege mit Hilfe einer Rasierklinge aufgeteilt und 15-20 Eier in Petrischalen für die Exposition und die jeweilige Kontrolle überführt. Die Schalen wurden in einem beleuchteten Klimaschrank (Hell/Dunkel 12h/12h) bei 27°C inkubiert und das Medium täglich erneuert. Um die Entwicklungsparameter zu erfassen, wurden alle Schnecken bis zu ihrem Schlupf einmal am Tag mit Hilfe eines Binokulars untersucht und das Ergebnis protokolliert. Als Endpunkte während der Embryonalentwicklung wurden festgelegt: (1) Zeitpunkt des erstes Auftretens der Augen, (2) Zeitpunkt des ersten Auftretens der Tentakel, (3) Herzschlagrate an Tag 9 und (4) Zeitpunkt des Schlupfs. Zusätzlich wurde (5) das Gewicht der Schnecken direkt nach dem Schlupf ermittelt.

4. Ergebnisse und Diskussion

4.1 Kapitel 1: Die Oocytenreifung bei *G. fossarum*

In den 12 Wochen des Experiments konnte ein vollständiger Zyklus der Eireifung bei *Gammarus fossarum* untersucht werden. Da die Tiere im Dezember, am Ende der Reproduktionspause, im Freiland gefangen wurden und annähernd dieselbe Größe hatten, verlief ihr Zyklus weitestgehend synchron. Zu beobachten war, dass zwischen der 3. und 4. Probenahme Praecopulastadien auftraten und die Weibchen reife Oocyten ins Marsupium ablegten. Dieser Zeitpunkt wurde als Ende bzw. Neuanfang des Zyklus definiert. Wie zu erwarten war, traten so genannte „*late vitellogenic oocytes*“ (LVO) am Ende des Zyklus auf, was in diesem Fall den Probenahmen eins und zwei entsprach. Dieser Zelltyp entsteht bei der Reifung aus „*early vitellogenic oocytes*“ (EVO) und unterscheidet sich von diesen durch vermehrte Einlagerung von Nährstoffen und daher einer stärkeren Kompartimentierung der Zellen. Da am Ende eines Zyklus die nicht zur Reifung gekommenen Oocyten atretisch und wieder absorbiert werden, müssen sie LVO in jedem Zyklus neu gebildet werden und treten deshalb auch erst in der zweiten Hälfte der Reifung auf. Der an sich sehr hohe Anteil an atretischen Oocyten in dieser Untersuchung ist mit hoher Wahrscheinlichkeit darauf zurückzuführen, dass sich die untersuchten Gammariden erst am Übergang zu geschlechtsreifen Tieren befanden und daher die Reifungsprozesse bei ihrem ersten Zyklus noch nicht optimal abliefen.

In den biochemischen Analysen wurden die beiden Stressproteine hsp70 und hsp90

untersucht, wobei hsp70 als Marker für allgemeine Schädigung der Integrität intrazellulärer Proteine herangezogen werden kann (Haslbeck, 2002; Höhfeld et al., 2001; Bukau & Horwich, 1998). Für Hsp90 hingegen ist bekannt, dass es bei Vertebraten eine wichtige Rolle bei der Modulation der Estrogen-Rezeptor-Wechselwirkung einnimmt (Pratt & Toft, 1997; Sabbah et al., 1996). Die Level beider Proteine wurden daher auf mögliche Veränderungen während der Eireifung hin untersucht. Die Ergebnisse zeigten, dass hsp70 und hsp90 einen entgegengesetzten Kurvenverlauf während des Entwicklungszyklus der Oocyten aufweisen. Zu Beginn des Zyklus, wenn neue Oocyten gebildet werden, steigt der hsp70 Level in den Gammariden an, was mit der vermehrten Proteinsynthese und der Aufgabe von hsp70 als Chaperon begründet werden kann. Zu diesem Zeitpunkt ist der Level an hsp90 entsprechend gering. Mit fortschreitender Reifung spielt die Proteinsynthese eine immer geringere Rolle und der hsp70 Level nimmt bis zu einem Minimum direkt vor dem Ablegen der Eier ins Marsupium ab. Da die Reifung vermutlich auch bei Gammariden ein hormongesteuerter Prozess ist, lässt sich damit auch der beobachtete Anstieg an hsp90 erklären, wenn man annimmt, dass hsp90 bei Gammariden, ähnlich wie bei Vertebraten, an diesen Vorgängen beteiligt ist.

Mit den Ergebnissen, die das Einhergehen von Oocytenreifung und biochemischen Parametern während der Oocytenentwicklung bei *G. fossarum* zeigten, konnte die Grundlage für Biomarkerstudien gelegt werden, die im weiteren Verlauf der Arbeit Einflüsse von hormonell wirkenden Substanzen auf Gammariden nachweisen sollten.

4.2 Kapitel 2: Der Einfluß von Einleitern im Freiland

Die Freilanduntersuchungen am Lockwitzbach und an der Körsch zeigten, dass sich die beiden Bäche hinsichtlich der Reaktion der Stressproteine deutlich voneinander unterscheiden. Für den Lockwitzbach ließ sich bei Tieren beider Größenklassen, weder für hsp70 noch für hsp90, ein einheitliches Muster erkennen, das auf einen Effekt des Einleiters schließen lassen könnte. An der Körsch hingegen konnte bei den Tieren der Größe L („*large*“) zu allen Probenahmezeitpunkten eine Erhöhung der Stressproteine (hsp70 und hsp90) an der Probestelle unterhalb des Einleiters festgestellt werden. Für hsp70 kann dies zum einen durch eine Stressreaktion, ausgelöst durch die Belastung des Einleiters, aber auch durch den Reifezustand der Tiere, der, wie in Kapitel 1 beschrieben, positiv mit hsp70 korreliert, erklärt werden. Demzufolge sollte sich der hsp90 Level umgekehrt verhalten und müsste an der Probestelle flußabwärts erniedrigt sein, was aber nicht der Fall ist. Der Einleiter scheint

demnach diese Vorgänge zu entkoppeln.

Bei den histologischen Endpunkten verhält es sich ähnlich. Am Lockwitzbach können keine Unterschiede zwischen den Probestellen oberhalb und unterhalb des Einleiters verzeichnet werden. Im Gegensatz zu den Stressproteinen werden die Effekte an der Körsch nur bei Tieren der Größenklasse M („*medium*“) deutlich. Im Jahr 2000 lag der ermittelte Reifeindex an der Körsch unterhalb des Klärwerks höher als oberhalb, zudem wies die größere Fläche der LVO's und das vermehrte Auftreten von Atresien an der unteren Probestelle ebenfalls darauf hin, dass die Entwicklung der Oocyten durch die im Einleiter vorhandenen Substanzen beschleunigt wurde. Gestützt wurde dies durch chemische Analysen, die eine Steigerung des estrogenen Potentials durch die Einleitung der Kläranlage nachwiesen. Dieses Potential war an der Körsch vergleichsweise höher als am Lockwitzbach, was auch erklärt, warum die Effekte des Einleiters vor allem an der Körsch deutlich zu sehen waren.

Mit den Ergebnissen aus den Jahren 2000 und 2001 wurde des weiteren eine Clusteranalyse durchgeführt. Berücksichtigte man dabei alle erhobenen histologischen und biochemischen Parameter (außer hsp70 für welches der Datensatz nicht vollständig vorlag) so resultierten vier Hauptcluster, die sich durch die vier Probenahmezeitpunkte charakterisieren ließen. Bei drei der vier Hauptcluster ließen sich mit dieser Analyse zusätzlich die beiden Bäche voneinander trennen. Eine Trennung der Probestellen in solche oberhalb bzw. unterhalb des Einleiters ließ sich mit diesem Verfahren jedoch nicht nachweisen. Berücksichtigte man bei der Clusteranalyse nur Tiere der Größenklasse M, so wurde eine Auftrennung in zwei Cluster (Frühjahr und Herbst) erreicht. Diese Befunde zeigen, dass jahreszeitliche Einflüsse eine erhebliche Auswirkung auf die Situation der Tiere haben und dass mögliche Effekte des Einleiters hierdurch z.T. überlagert werden können. Als Konsequenz für diese Untersuchung bedeutet das, dass Muster oder Trends bezüglich der Lage der Probestelle oft nicht durchgängig zu erkennen sind. Für zukünftige Freilandstudien ist dies eine sehr wichtige Hintergrundinformation, die bereits in die Planung solcher Untersuchungen eingehen sollte.

4.3 Kapitel 3: BPA-Exposition in Mesokosmen

Durch die Exposition von *G. fossarum* in den Fließrinnen war es möglich, äußere Faktoren wie z.B. Temperatur, Nährstoffversorgung und die Wasserqualität konstant zu halten, um die durch Bisphenol A verursachten Effekte isoliert zu betrachten. Bis zu einer Versuchsdauer von 69 Tagen kann davon ausgegangen werden, dass die Haltungsbedingungen keinen

Einfluss auf die Gammariden hatten. Bei der letzten Probenahme, 103 Tage nach der Exposition, konnte eine erhöhte Mortalität in den Rinnen, einschließlich der Kontrollrinne, festgestellt werden.

Die Erhöhung der Wassertemperatur in der Fließrinne von 15°C auf 17°C hatte im Verlauf des Experiments einen Anstieg des hsp70 Levels zur Folge, zudem konnte eine Beschleunigung der Oocytenreifung festgestellt werden. Diese Veränderungen sind auf Grund beschleunigter physiologischer Vorgänge bei erhöhter Temperatur nicht erstaunlich, dass diese aber bereits bei einer Differenz von 2°C und einer relativ kurzen Expositionszeit zu erkennen waren, ist für eine zukünftige Interpretation von Freilanddaten eine wichtige Erkenntnis.

Ähnlich wie in Kapitel 2 beschrieben, wiesen die Ergebnisse der histologischen Untersuchungen auch bei der Exposition gegenüber einer einzigen Substanz, BPA, in die gleiche Richtung: BPA beschleunigte in allen drei getesteten Konzentrationen die Reifung der Oocyten in der Gonade, was sich auch in einer Erhöhung des Reifeindex widerspiegelte. Eine ähnliche Reaktion auf estrogen wirkende Substanzen ist in der Literatur bereits für mehrere Arten beschrieben (Duft et al., 2003; Oehlmann et al., 2000; Andersen et al., 1999). Mit der beschleunigten Reifung ging die dosisabhängige Abnahme an atretischen EVO's und deren Zellfläche im Querschnitt einher.

Die Analyse der hsp70 Stressproteine zeigte keine einheitlichen Effekte, sowohl bei niedrigen als auch bei hohen Konzentrationen von BPA. Im Gegensatz dazu konnte eine Reduktion des hsp90-Levels bei allen BPA-exponierten Tieren festgestellt werden. Da hsp70, das als interne Kontrolle für unspezifischen Stress dienen sollte, keine quantitativen Veränderungen zeigte, ist davon auszugehen, dass die Effekte auf hsp90 spezifisch durch BPA hervorgerufen wurden. Da Studien weitgehend fehlen, welche die Wirkung von EDC's auf Crustaceen mechanistisch erklären könnten (de Fur, 1999), können diese Ergebnisse nur mit Beobachtungen an Vertebraten verglichen werden. Im Uterus von Mäusen führte BPA zu einer dosisabhängigen Veränderung des hsp90-Levels (Papaconstantinou et al., 2001). Unklar blieb allerdings, warum der hsp90 Level in den Studien mit *G. fossarum* sank und nicht, wie in anderen Arbeiten beschrieben, durch die Zugabe von BPA anstieg (Shyamala et al., 1989; Ramachandran et al., 1988). Obwohl diese Ergebnisse gegensätzlich sind, so entsprechen die Ergebnisse der Mesokosmos-Experimente den Daten, die im Freiland erhoben wurden (Kapitel 2); hier wurde ebenfalls eine Reduktion des hsp90-Levels der Gammariden bei Erhöhung des estrogenen Potentials durch einen Kläranlagenausfluß festgestellt.

4.4 Kapitel 4: Intersex bei *G. fossarum*

Intersex ist ein Phänomen, welches bei Crustaceen häufig vorkommt (Munro, 1953; Ginsburger-Vogel, 1975; Legrand et al., 1987; Moore & Stevenson, 1991; Sangalang & Jones, 1997), für die Art *Gammarus fossarum* jedoch erstmals durch diese Arbeit beschrieben wurde. Bei Intersexen treten an demselben Individuum sowohl weibliche wie auch männliche Geschlechtsmerkmale auf. In den Freilanduntersuchungen an der Körsch und am Lockwitzbach (Kapitel 2) konnten Intersex-Tiere nachgewiesen werden, die Häufigkeit mit der dieses Phänomen in den jeweiligen Populationen auftrat unterschied sich jedoch erheblich voneinander. An der Körsch waren 0,6% aller Gammariden Intersex-Tiere, am Lockwitzbach wiesen 8,8% Merkmale beider Geschlechter auf, wobei sich die Häufigkeit innerhalb der Bäche zwischen den Probestellen zum Teil signifikant unterschied. Bei allen Intersex-Tieren waren Oostegite als sekundäre weibliche Geschlechtsmerkmale vorhanden, welche bei Weibchen das Marsupium bilden. Zudem konnten ein oder zwei Penispapillen als typische männliche Merkmale gefunden werden. Die histologischen Auswertungen zeigten, dass die Gonaden aller untersuchten Tiere eindeutig weiblich waren und kein männliches Gonadengewebe bzw. Spermien nachweisbar waren. Die Tatsache, dass einige Intersex-Tiere in der weiblichen Praecopulastellung gefunden wurden und zudem Eier im Marsupium auftraten, lässt darauf schließen, dass es sich bei Intersexen in diesen Fällen um funktionelle Weibchen handelte. Diese Ergebnisse stellten die Grundlage für weitere Untersuchungen dar, die ermitteln sollten, ob das Intersex-Phänomen bei *G. fossarum* induzierbar bzw. reversibel ist (Jungmann et al., 2004).

4.5 Kapitel 5: Ein Estrogenrezeptor α-ähnliches Protein bei *G. fossarum*

Seit langem wird angenommen, dass Steroidrezeptoren ihren Ursprung bei den frühen Deuterostomiern haben (Thornton, 2001) und bis zum Jahre 2002 konnte auch kein Rezeptor außerhalb dieses Taxons nachgewiesen werden. Durch unsere Untersuchungen an *Gammarus fossarum* konnte, mit Hilfe eines Immunoblots ein Estrogenrezeptor α (ERα)-ähnliches Protein in weiblichen Tieren nachgewiesen werden. Dieses Protein mit einem Molekulargewicht von ca. 62 kD trat ausschließlich bei adulten Weibchen auf, bei Männchen und juvenilen Tieren war es nicht vorhanden. Als Positivkontrolle diente in diesem Versuch Uterusgewebe der Ratte, als Negativkontrolle Kiemengewebe von Tilapia. Um Kreuzreaktionen mit anderen nuklearen Rezeptoren, wie z.B. dem Ecdysonrezeptor von Insekten ausschließen zu können, wurden zusätzlich Proben aus Fliegenlarven (*D.*

melanogaster) und den Beinen von Schaben (*P. americana*) untersucht, wobei bei keinem eine Kreuzreaktion des Antikörpers festgestellt wurde. Dieses Ergebnis deckt sich mit Befunden aus anderen Studien, die ebenfalls von der Präsenz eines Estrogenrezeptors bei Protostomiern berichten (di Cosmo et al., 2002; Thornton et al., 2003).

Durch den Einsatz von degenerierten Primern und mit Hilfe einer RT-PCR konnte ein 96bp langes Stück der cDNA von *G. fossarum* Weibchen amplifiziert und abschließend sequenziert werden, das eine hohe Homologie zu ER Genen verschiedener Amnioten aufwies (81% - 90%). Die Homologie zu bekannten Sequenzen aus Fischen und Schnecken war mit 65% bzw. 58% deutlich geringer und nahm beim Vergleich zu den nuklearen Rezeptoren von Arthropoden weiter ab (49%). Es scheint, als ob die Teilsequenz des ERα-ähnlichen Gens von Gammariden zumindest strukturell eher derjenigen von Tetrapoden als den Rezeptorengenen taxonomisch näher verwandter Taxa ähnelt. Ähnliches wurde kürzlich für eine Teilsequenz des ER-Rezeptorgens von *Marisa cornuarietis* bekannt (S. Jobling, unveröffentlicht).

In einem weiteren Versuch wurde die Induzierbarkeit des ERα-ähnlichen Proteins durch 17α-Ethinylestradiol (EE2) getestet. Es stellte sich heraus, dass nach einer Exposition von 5 Tagen gegenüber 10 µg EE2/L der ERα-Level ausschließlich bei adoleszenten Weibchen zugenommen hatte. Diese erreichten ein Niveau vergleichbar dem adulter Weibchen. Männchen, sowie adulte Weibchen zeigten keine Reaktion auf die estrogene Belastung. Die Präsenz und mögliche Induzierbarkeit eines ER könnte eine mechanistische Erklärung für Effekte, die bei Gammariden durch den Einfluss estrogener Substanzen bereits beschrieben wurden (Duft et al., 2003; Pascoe et al., 2002; Watts et al., 2002; Kirkebride-Smith et al., 2001; Watts et al., 2001b), liefern.

4.6 Kapitel 6: Ein Embryotest mit Marisa cornuarietis

Im ersten Ansatz zur Etablierung eines Embryotests wurden Eier von *Marisa cornuarietis* gegenüber zwei Konzentrationen an Cadmium (250 µg/L und 500 µg/L) exponiert. Dieses Schwermetall wurde ausgewählt, weil es erwiesenermaßen Auswirkungen auf die Embryonalentwicklung anderer Organismen hat (Cœurdassier, 2003; Gomot, 1998; Itow et al., 1998; Eaton et al., 1978) und eingesetzt, um entwicklungsrelevante Endpunkte für den Embryotest zu definieren. Eine Mortalität vor dem Schlupf von über 90%, wurde bereits durch 500 µgCd/L erreicht, einer Konzentration, die z.T. wesentlich niedriger als in der Literatur beschriebene Werte für *Danio rerio* Embryonen im etablierten DarT-Test (Hallare et al., 2005) liegt, was somit die hohe Sensitivität des Versuchsorganismus wider spiegelt. Im

Ansatz mit 250 µgCd/L konnte eine Verzögerung in der Ausbildung der Augen und der Tentakel gegenüber der Kontrolle und darüber hinaus eine Gewichtsreduktion der Tiere nach dem Schlupf festgestellt werden. Der stärkste Effekt bestand in einer Verzögerung des Schlupfs bei cadmiumbelasteten Schnecken.

17α-Ethinylestradiol diente in dieser Untersuchung als Positivkontrolle für Substanzen mit estrogener Wirkung. Im Embryotest zeigte sich eine signifikante Abnahme der Herzschlagrate gegenüber der Kontrolle; derselbe Effekt trat allerdings auch in der Lösungsmittelkontrolle auf, wenn auch in abgeschwächter Form. Das höchste Gewicht nach dem Schlupf wurde ebenfalls in den EE2-behandelten Schnecken gemessen. Der Schlupf fand zwischen Tag 11 und 14 statt, wobei sich eine Tendenz zu einer beschleunigten Reifung und zu einem früheren Beginn des Schlupfs abzeichnete. Dieses Ergebnis stimmt mit anderen Studien, die von einer gesteigerten Reproduktionrate nach EE2-Behandlung berichten überein, was sich wiederum in erhöhten Eizahlen oder in einer Vergrößerung der Population widerspiegelte (Watts et al. 2002, Andersen et al. 1999).

Bisphenol A als potentiell endokriner Disruptor wurde in den Konzentrationen 50 µg/L und 100 µg/L im Embryotest mit *M. cornuarietis* eingesetzt. Hierbei konnte eine dosisabhängige Abnahme der Herzschlagrate festgestellt werden. Des weiteren wurde mit zunehmender BPA-Konzentration ein Anstieg des Gewichtes frisch geschlüpfter Schnecken beobachtet, was den Ergebnissen für die Positivkontrolle mit EE2 entsprach. Nach 11d Exposition entsprach die Schlupfrate ebenfalls den Verhältnissen der Positivkontrolle mit EE2. Auswirkungen von endokrinen Disruptoren auf *M. cornuarietis* wurden bereits schon früher beschrieben (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2000) allerdings wurde hierbei die Embryonalentwicklung ausgeklammert. Die Tatsache, dass sich die für BPA erzielten Ergebnisse größtenteils mit denjenigen der Positivkontrolle decken, zeigt, dass es möglich ist, mit diesem Testprotokoll endokrin wirksame Substanzen zu identifizieren. Da ein dem etablierten *Danio rerio* Embryotest (DIN 38415) vergleichbares Protokoll für Invertebraten bislang noch nicht vorlag, könnte dieser neuentwickelte Test einen vielversprechenden Ansatz für den Einsatz in der ökotoxikologischen Routine im aquatischen Bereich darstellen.

Schlussfolgerungen

Die Reifung der Oocyten bei *Gammarus fossarum* wird von zyklischen aber einander entgegengesetzten Verläufen der Level von hsp70 und hsp90 begleitet, was als Grundlage für

den weiteren Einsatz dieser Parameter als Biomarker diente. Es stellte sich heraus, dass die untersuchten Biomarker sowohl im Einzelsubstanz-Experiment als auch in komplexer Freilandsituation, eine einheitliche Reaktion auf die Belastung mit endokrin wirkenden Substanzen zeigen. Generell scheint durch ein erhöhtes estrogenes Potential die Reifung der Oocyten beschleunigt und die Zyklen dadurch verkürzt zu werden, um eventuell potentiell eine Erhöhung der Reproduktion zu erreichen. Die Rolle von hsp90 bei der Modulation der Steroidwirkung bei Invertebraten bleibt weiterhin unklar, jedoch wird durch den Nachweis eines ERα-ähnlichen Proteins bei *G. fossarum* eine mögliche Beteiligung an hormonell gesteuerten Prozessen gestützt. Auch wenn der letzte Nachweis durch die vollständige Sequenzierung des ERα Gens noch erbracht werden muss, so deuten die vorliegenden Ergebnisse stark darauf hin, dass bei weiblichen *G. fossarum* ein solcher Rezeptor vorhanden ist.

Auch wenn die Problematik von endokrinen Disruptoren in der Umwelt in den letzten Jahren zunehmend an Aufmerksamkeit gewonnen hat, so ist das Wissen ebenso wie die Forschungsaktivitäten hinsichtlich der Wirkungen auf Invertebraten noch immer sehr gering. Durch die Erstellung eines Protokolls für einen Embryotest mit *Marisa cornuarietis* konnte gezeigt werden, dass endokrine Effekte nicht nur in adulten Tieren nachzuweisen sind, sondern schon sehr früh in der Ontogenese. Mit dieser für einen Routinetest vergleichsweise sensitiven Methode können die Möglichkeiten der Ökotoxikologie erweitert oder ergänzt werden, um potentielle endokrine Disruptoren und ihre Wirkungen in der Umwelt zu detektieren.

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Eigenanteil an den durchgeführten Arbeiten in den zur Dissertation eingereichten Publikationen und Manuskripten:

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Kapitel 2

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Eingebunden in das Projekt "Xehogamm" des Umweltbundesamts. Hälftige Beteiligung an der Versuchsdurchführung und Erhebung des biochemischen und limnologischen Teils. Kompletter Eigenanteil an der Auswertung. Fachliche Betreuung durch Prof. Dr. H.-R. Köhler und PD Dr. R. Triebskorn (Universität Tübingen).

Kapitel 3

Schirling M., Jungmann D., Ladewig V., Ludwichowski K.-U., Nagel R., Köhler H.-R., Triebskorn R. (in press) Bisphenol A in artificial indoor streams: II. Stress response and gonad histology in *Gammarus fossarum* (Amphipoda). Ecotoxicology.

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Kapitel 4

Ladewig V., Jungmann D., Schirling M., Triebskorn R., Nagel R. (2002) Intersexuality in *Gammarus fossarum* Koch, 1835, (Amphipoda). Crustaceana 75(11). 1289-1299.

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Kapitel 5

Köhler H.-R., Schirling M., Triebskorn R., Nagel R., Schönfelder G. (to be submitted) Estrogen receptor was not lost at the basis of ecdysozoan evolution: Expression in Crustacea is stimulated by 17α-ethinylestradiol

Beteiligung an der Versuchsplanung, kompletter Eigenanteil an der Durchführung und Auswertung der proteinbiochemischen Untersuchungen. Fachliche Betreuung durch Prof. Dr. H.-R. Köhler (Universität Tübingen).

Kapitel 6

Schirling M., Bohlen A., Triebskorn R., Köhler H.-R. (submitted) An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential endocrine disruptors. Aquatic Toxicology

Kompletter Eigenanteil bei der Versuchsplanung und des Aufbaus. Versuchsdurchführung zusammen mit A. Bohlen. Kompletter Eigenanteil an der Auswertung. Fachliche Betreuung durch Prof. Dr. H.-R. Köhler und PD Dr. R. Triebskorn (Universität Tübingen).

Kapitel 1

VARIATION IN STRESS PROTEIN LEVELS (HSP70 AND HSP90) IN RELATION TO OOCYTE DEVELOPMENT IN *GAMMARUS FOSSARUM* (KOCH 1835)

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SUMMARY

Variations in the level of stress proteins (hsp70 and hsp90) were measured during the reproductive cycle of the amphipod crustacean, *Gammarus fossarum* (Koch 1835). The reproductive cycle was determined histologically while the heat shock protein-levels were measured by an immunoblotting assay. Gammarids of defined body length were kept in a glass aquarium for 12 weeks under constant conditions. Animals were removed every 14 days for investigation. Histological investigations showed that the maturation stage of the female gonad could be identified according to the structure of the oocytes, and that an almost complete reproductive cycle occurred within 12 weeks. Hsp70 and hsp90 levels were found to be inversely correlated over the course of the reproductive cycle. At the beginning of the reproductive cycle, the hsp90 level was found to be low while that of hsp70 was at its peak, whereas, at the end of the cycle, when mature oocytes are present, the opposite was true. The data indicate that the levels of stress proteins reflect the maturity stage of the oocytes. This finding provides prerequisite baseline information to interpret biomarker studies on endocrine effects of chemicals in gammarids.

Key words: Amphipoda, *Gammarus fossarum*, hsp70, hsp90, oocyte development

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Invertebr. Repr. Dev. (2004) 45(2). 161-167

INTRODUCTION

Recent studies have highlighted the effects of xenohormones on invertebrate species (Andersen et al., 2001; Panter et al., 2002). Watts and co-workers (2002) reported that exposure to 17 α-ethinylestradiol (EE2) caused a drift in the sex ratio of *Gammarus pulex* and an increase in population size due to recruitment of neonates and juveniles. Even though these effects have been described, the underlying mechanism has not yet been identified in these animals.

In contrast, the influence of endocrine disrupting chemicals on vertebrates is well established and proven by several studies (e.g., Colburn, 1995; Fox, 2001; Schreurs et al., 2002). To enable the binding of an estrogen to the estrogen receptor (ER), the receptor must be available in a distinct way. The activated form of the ER is a multi-protein complex that contains different molecules like hsp70 and hsp40, Hip, p23 and Hop, either for the stabilization of the complex itself or the binding of the hormone. Furthermore, the molecular chaperone hsp90 interacts with non-ligand-bound steroid hormone receptors and regulates their activity. The receptor–hormone complex is able to bind to DNA at the estrogen response element (ERE) site and initiates protein synthesis. This modulation of the ER seems to explain the fact that the level of soluble hsp90 can be correlated with the activation of the steroid hormone receptors, at least in vertebrates (Sabbah et al., 1996). In contrast to hsp90, hsp70 is induced by increasing amounts of unfolded, nascent polypeptide strains (which may occur during the formation and development of new oocytes) as well as by misfolded proteins (which may occur during oocyte degradation) (Edington et al., 1989). Detailed information on the manifold cellular processes hsps are involved in can be obtained from Haslbeck (2002), Höhfeld et al. (2001) and Bukau and Horwich (1998).

This study investigated the relationship between hsp70, hsp90 and the developmental status of the oocytes. The reproductive cycle of *G. fossarum* is well known and has been described in detail by Pöckl (1992). Since the development of oocytes in invertebrates may be a steroid-controlled process, it was hypothesized that variations in the hsp levels would coincide with the reproductive cycle of gammarids. The results from the present histological investigation coupled with biochemical analysis may provide baseline information to interpret future findings in field studies and may help to establish a biomarker for substances interfering with the reproductive system in invertebrates.

MATERIAL AND METHODS

Gammarids were collected in December 2000 from an unpolluted stream in SW Germany (Goldersbach near Tübingen). Specimens with a body length of precisely 6 mm were selected for further investigation in the laboratory. According to Pöckl et al. (1990), gammarids with a 6 mm size have reached sexual maturity. The experimental design, together with the sampling time in December, which is the start of the reproductive period for the next year for *G. fossarum*, ensured that the majority of the sampled animals were at an identical developmental stage at the beginning of the investigation.

A total of 323 specimens were held in a 240-L glass aquarium containing a well-aerated water mixture of 2/3 tap water and 1/3 water from the Goldersbach stream. The aquarium was filtered by an external filter which also provided a moderate flow inside.

During the 3-month experimental period, the conditions were held constant at 12°C water temperature and a photoperiod of 14 h (light):10 h (dark). Food obtained predominantly from the sampling site was applied *ad libitum*.

Stress protein analysis

For the stress protein analysis, 8 animals from the aquarium were collected randomly every 14 days over a period of 12 weeks (samplings 1 to 6). Firstly, the animals were sexed according to Heinze (1932) and Schellenberg (1942). Female gammarids possess four pairs of brood plates (oostegites) in the thoracic region of the body. Males bear two penial papilla (the end of the v*as deferens*) between the last pairs of walking legs.

After shock freezing and homogenization in an extraction buffer [80 mM potassium acetate, 4 mM magnesium acetate, 20 mM Hepes, 2% protease inhibitor cocktail (Sigma P8340), pH 7.5], the samples were centrifuged (12 min, 20,000 g at 4°C). Total protein concentration in the supernatant was determined according to the method of Bradford (1976). Constant amounts of protein (40 µg of total protein per lane) were subjected to SDS-PAGE (12% acrylamid-bisacrylamid) for 20 min at 80 V and 120 min at 120 V in duplicate (hsp90 and hsp70 were run on separate gels). Protein was transferred to nitrocellulose by semi-dry blotting, and the filter was blocked for 2 h in 50% horse serum in Tris-buffered saline (TBS: 50 mM Tris, 150 mM NaCl pH 7.5). After washing in TBS, monoclonal antibody (mouse anti-human hsp70; Dianova, Hamburg, Germany, dilution 1:5,000 in 10% horse serum/TBS, or mouse anti-water mold hsp90; StressGen Victoria, Canada, dilution 1:800) was added, and

incubated at room temperature overnight. After washing in TBS for 5 min, the nitrocellulose filters were incubated in the second antibody (peroxidase- conjugated goat anti-mouse IgG Dianova, Germany, dilution 1:1,000 in 10% horse serum/TBS) at room temperature for 2 h. After repeated washing in TBS for 5 min, the antibody complex was detected by 1 mM 4-chloro(1)naphtol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol. The grey scale values of the Western blot protein bands were quantified using a densitometric image analysis system (Herolab E.A.S.Y., Germany). The linear relationship between hsp concentration and the signal density was previously described by Schill et al. (2002).

Histological analysis

Animals were sampled and their sex determined until 8 females (n = 8) per sampling were available for histological analysis. Subsequently, they were decapitated and fixed in a 2% glutardialdehyde solution (dissolved in 0.005 M cacodylate buffer). Prior to the embedding the gammarid samples were decalcified with four applications of 5% trichloroacetic acid for two days. They were then dehydrated in a graded series of ethanol and finally embedded in Technovit (Heraeus Kulzer, Germany). Then the animals were sectioned as follows: per individual, eight series each of 16 sagittal sections (4 µm thickness) were cut on a Reichert Jung 2050 microtome. The arrangements of the eight section series were dependent on the individual width of each specimen assuring a complete overview over the histology of each. Sections were stained with Methylene blue-Azur II according to Richardson et al. (1960) and examined using a light microscope (Zeiss, Axioscop 2).

The females (n = 8 per sampling) were classified based on the maturity status of their gonads. This was accomplished by examining the oocyte stages present in the ovary: the most proceeded oocyte which did not show any atresia was considered to define the developmental stage of the animal. In this work four different stages are distinguished on the basis of the work of Tan-Fermin and Pudadera (1989), as shown in Fig. 1:

- Early vitellogenic stage: small basophilic cells with few vesicles in the cytoplasm; occurrence of perinuclear heterochromatin.
- Late vitellogenic stage: larger, cuboidal cells with large vacuoles of lipids or proteins; follicle cells surrounding the membrane.
- Mature stage: this is the last stage of development before oocytes are released to the marsupium; the characteristic egg membrane surrounds the cell, and the ellipsoidal nucleus is located next to the membrane.

- Spent stage: only oocytes which are not released into the marsupium belong to this stage; the oocytes become atretic and undergo lysis.

Since it was possible to distinguish whether atretic oocytes were derived from either early vitellogenic stages (EVO) or late vitellogenic stages (LVO), intact and atretic oocytes were recorded separately for EVO and LVO stages.

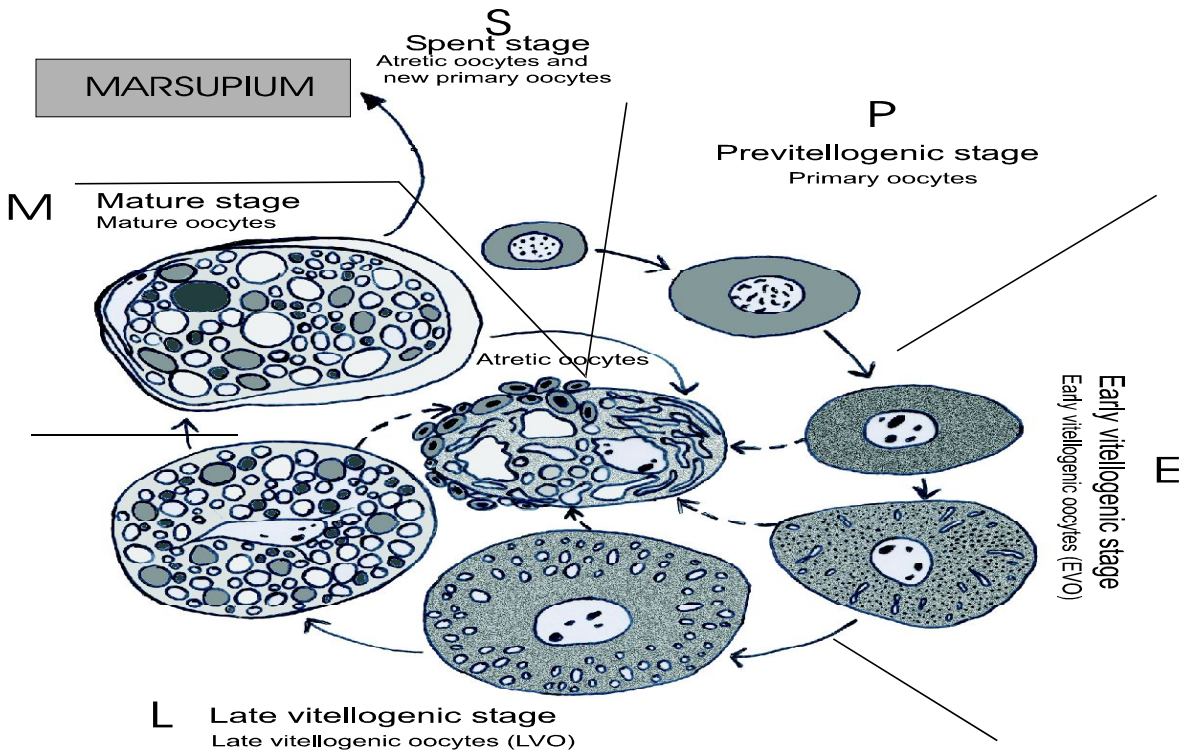

Fig.1. Characteristics of different maturity stages of *Gammarus fossarum* (modified after Tan - Fermin and Pudadera, 1989).

Regression analysis and statistics

Polynominal regression analysis, analysis of variance and the Wilcoxon-Mann-Whitney *R*-test or Student's *t*-test were conducted with JMP statistical software (SAS).

RESULTS AND DISCUSSION

General observation

None of the animals placed in the aquarium at the beginning of the experiment was paired in the so-called precopula phase. Five to six weeks later the vast majority of individuals were in this phase, corresponding to the normal situation in the field at this time. Between the end of December and early January, the reproductive break ends and precopula pairs are found more or less simultaneously. This behavior is triggered in the entire population by abiotic factors such as photoperiod. This event coincides with sampling event no. 3 in the experiment. Two weeks later, at the time of sampling no. 4, the majority of the females showed oocytes in their brood pouch (marsupium). This observation, together with histological data, provided reliable information about the reproductive stages of the specimens.

Histology

The six samples, taken every 14 days over a 3-month period, provided an overview of the maturation of the oocytes. In this investigation, the "beginning" of the cycle was identified shortly after sampling no. 4 when the eggs were released to the marsupium. The precopula stage and the release of the mature oocytes into the brood pouch between sampling three and four were considered as the end of the reproductive cycle.

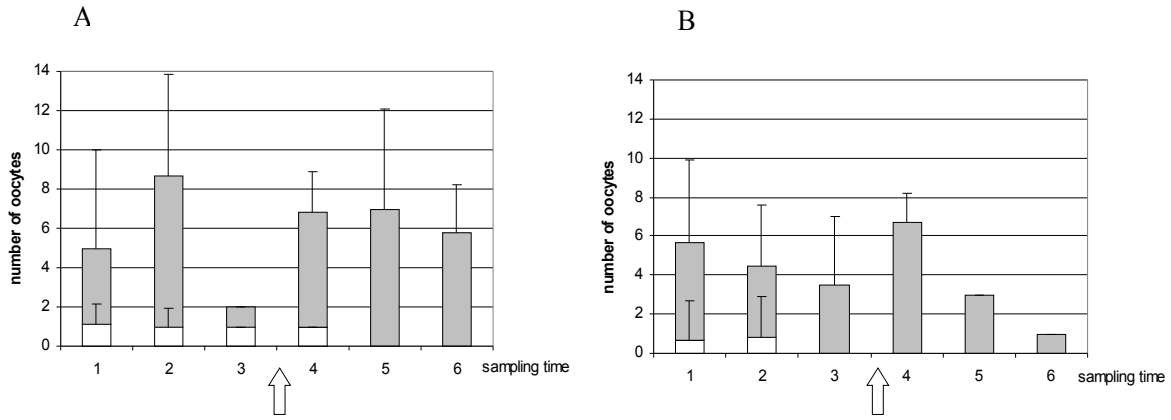

Fig. 2. Mean number and standard deviation of atretic (shaded column portions) and intact oocytes (A, EVO; B, LVO) at each sampling time. Arrow: Mature oocytes were released into the marsupium. No error bars are shown when replicate numbers were too low (n = 1–2).

Fig. 2 illustrates the mean numbers of intact EVO as well as atretic EVO during the cycle. From sampling no. 1 to sampling no. 4, a small number of intact cells regularly occured in the gonads. The number of atretic cells was found to be high in all stages except for the time when animals were in the precopula stage. Significant differences could not be found between the samplings.

The high number of atretic oocytes is probably due to the young age of the gammarids. We assume that animals with a length of 6 mm are in one of their first reproductive cycles. At this point it can be hypothesized that developmental processes may not work accurately in all oocytes and, therefore, the number of intact cells can be far lower than that of the atretic cells. Furthermore, the conditions in the aquarium were probably suboptimal in comparison to the situation in the field. In addition, the number of cells in the early stages of the cycle is always higher than that of cells close to maturation, even in old animals (Schirling, unpublished).

Intact LVO were limited to the second half of the experimental period as shown in Fig. 3, again with a relatively high number of atretic cells.

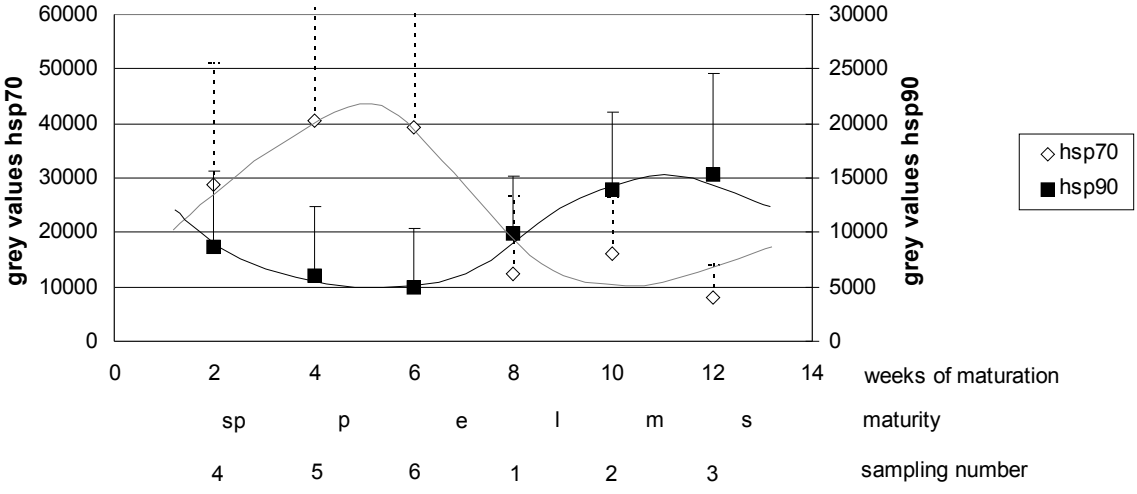

Fig. 3. Mean hsp90 and hsp70 levels and standard deviations for the maturity stages of *G. fossarum*. Lines represent best thirddegree polynomial fit. p, previtellogenic stage; e, early vitellogenic stage; l, late vitellogenic stage; m, mature stage; s, spent stage; sp, intermediate stage between spent stage and the previtellogenic stage of the next cycle of oocyte maturation.

Stress proteins

The hsp90 level, measured in grey scale values, shows an increase from sampling no. 1 to sampling no. 3 (Fig. 3). Beyond this time, and the associated precopula stage, the levels decrease again over the next three samplings, reflecting the beginning of the following reproductive cycle. All hsp90 levels of the first half of the cycle (sampling nos. 4, 5 and 6) are lower than at the end of the maturation of the oocytes (nos. 1, 2 and 3). The best polynomial fit of the third degree resulted in a regression curve with the equation [hsp90] =20,160- 7,793.08 [weeks] +1,198.45 [weeks]^2 - 48.49 [weeks]^3 and an R of 0.985. Analysis of variance revealed significance at the $P = 0.005$ level.

The hsp70 level followed a course that was inversely correlated to that of hsp90. At the beginning of the reproductive cycle (sampling nos. 4, 5 and 6), the grey scale values are much higher than towards the end (nos. 1, 2 and 3). The lowest level here was reached during the precopula phase at sampling no. 3 and increased from samplings 4 to 6. A third-degree polynomial resulted in the best fit (R = 0.924) using the equation [hsp70] = -9m319 + 27m628.1 [weeks] - 4,591.06 [weeks]^2 + 201.79 [weeks]^3. Despite the obvious negative correlation of the hsp70 and hsp90 (analysis of variance: P = 0.013), analysis of variance failed to show significance for hsp70 alone.

It is well known that, at least in mammals, hsp90 is induced by steroid hormones (Shymala et al., 1989) and that the level changes according to the level of hormones (Patchev et al., 1994). If the gestation of the oocytes inside the gonad is regulated by steroid hormones, our measurements reflect the progress of maturation. An increase of the hsp90 level towards sampling 3 would be expected, if steroid receptors are dependent on the hsp90 dimer. At the beginning of a new reproductive cycle there is no need for gestation of oocytes, and the creation of new oocytes has the immediate priority. This results in a decreasing level of hsp90.

The major task of hsp70 is to assist the correct folding of proteins during their synthesis. This could be the reason for the observed course of hsp70 during oocyte gestation. At later stages of maturation, when LVO and mature oocytes occur, the main activity inside the gonad is the storage of yolk vacuoles and the development of the eggsack. An enhanced intracellular production of proteins does not take place at this time. After the spent stage, when the eggs are released, the major activity is production of a new generation of previtellogenic and early vitellogenic oocytes. Therefore, for the fast growth of the oocytes, increased protein synthesis together with a higher induction of hsp70 is necessary, primarily in the early stages of the reproductive cycle.

Gender-specific variations

Prior to stress protein analysis, the gender of all specimens was determined to analyze whether or not hsp levels varied in a gender-specific way. Therefore, the dataset was divided according to the sex of the animals and the two subsets compared with each other. This was done separately for each sampling as well as combined for all samplings (Fig. 4).

In both cases, the pattern of hsp70 and hsp90 levels did not show any significant differences between male and female gammarids (Student's *t*-test). The specific trend of both heat shock protein levels is apparently the same during the reproductive cycle of males and females.

The variation in the stress protein levels in females was explained by the maturation of the oocytes. In the case of male gammarids, two possible mechanisms could explain the observed variation in the hsp levels. On one hand, the cycle could be due to spermatogenesis, which takes place in the gonads under the influence of the androgenic gland. This gland is located in a pouch of the *vas deferens* at the seventh pereiomer (Schmitz, 1992). Unfortunately, in contrast to oocyte maturation, little is known about spermatogenesis, but a mechanism analogous to that in the female seems likely. On the other hand, the observed cycle of hsp70 and hsp90 could be caused by the moulting hormone ecdysone. In crustaceans, ecdysone initiates ecdysis and is secreted to the haemolymph immediately before moulting. In between moults it is found only in low concentrations (Okumura et al., 2000). Hsp90 could be subject to ecdysone modulation and thus undergoes the same cycle (Chang et al., 1999). These results can help in the interpretation of field data where it is usually not possible to determine the gender of the gammarids during sampling (except for the precopula stages).

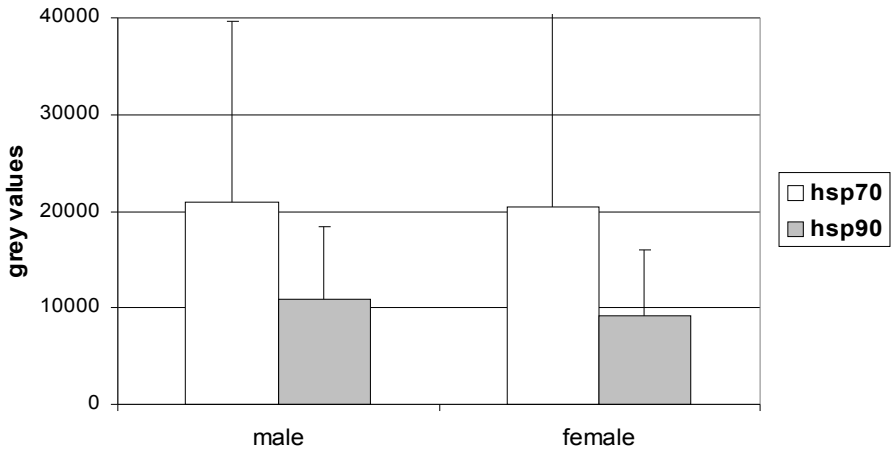

Fig. 4. Gender-specific variation of the hsp90 and hsp70 levels over all samplings, showing means and standard deviations.

CONCLUSIONS

The intention of the present study was to provide baseline data for the use of biochemical and histological biomarkers in the amphipod *G. fossarum*. For a more reliable interpretation of the effects of chemicals with endocrine potentials, it is necessary to know about the natural variability of parameters associated with the endocrine system. It was shown that the biochemical parameters hsp90 and hsp70 reflect the maturation of the oocytes. The controlled conditions, i.e., constant temperature, food, photoperiod and water conditions ensured that the changing levels could only be attributed to internal processes. We propose that the observed variation could be explained predominantly by the endogenously regulated development of the oocytes.

The selected time-frame of the experiment starting with the late stage of the reproductive break led to an almost perfect synchronization of the precopula stage. Thus, it was possible to observe a defined point in the reproductive cycle for most of the gammarids which allowed for an integrated interpretation of the biochemical and histological results. With one additional sampling and probably an additional focus on the previtellogenic oocytes, the reproductive cycle could have been entirely recorded, and valid predictions about what happens between sampling no. 6 and a putative sampling no. 1 of the next cycle would have been possible.

Regarding biomarkers, the investigated relationship between hsp90/70 levels and oocyte development may be useful tools for the prediction of the activity of endocrine acting chemicals. As mentioned in the introduction to this paper, gammarids must be subject to endocrine disruption, since a shift of the sex ratio towards female in populations of adult *G. pulex* was observed as a result of environmentally relevant concentrations of 17α-ethinylestradiol (Watts et al., 2002). Even though the underlying mechanism remains unclear for gammarids, these organisms should be regarded as useful biomonitors in this context.

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Kapitel 2

ENDOCRINE EFFECTS IN *GAMMARUS FOSSARUM* (AMPHIPODA): INFLUENCE OF WASTEWATER EFFLUENTS, TEMPORAL VARIABILITY AND SPATIAL ASPECTS ON NATURAL POPULATIONS

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ABSTRACT

In a field study, individuals of autochthounous populations of the amphipod *Gammarus fossarum* were examined for their maturity status, oocyte development, and biochemical parameters associated with their reproductive cycle. Variability in these parameters was related to abiotic exposure parameters varying in accordance to wastewater discharges, stream identity, and time. Patterns of all investigated parameters showed predominantly seasonal rather than spatial influence for both exposure and biologic effects. Single selected-effect parameters, however - such as the maturity index, late vitellogenic oocyte size and atresia, and the hsp90 level - responded to a sewage treatment plant discharge showing an estrogenic potential and also correlated significantly with the concentration of potential xenoestrogens at the different locations.

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INTRODUCTION

Endocrine disruption in wildlife has become a common object of research in recent years. For many substances, an endocrine disrupting effect has been demonstrated, and for even more substances this capacity is presumed. At the end of the 1970s, failure in reproduction of ospreys were one of the first phenomena linked to xenoestrogens (Spitzer 1978); other studies – such as those by Mason et al. (1986), Fry et al. (1987), or Guillette et al. (1994) – followed. To date, research is still mainly focused on vertebrate species, and little is known about the underlying mechanisms outside this subphylum with the exception of prosobranch snails (Oehlmann et al. 1996).

In other invertebrate phyla, recent studies have shown effects of potential endocrine disruptors on reproductive parameters or sex ratios (Pascoe et al. 2002; Kirkebride-Smith et al. 2001; Watts et al. 2001). In these studies, mostly laboratory tests using static or flow-through systems have been applied (Duft et al. 2003; Watts et al. 2002; Andersen et al. 2001). In this context, a strong impact of the artificial estrogen, 17α-ethinylestradiol, has been described on the sex ratio in cultures of freshwater crustaceans of the genus *Gammarus* (Watts et al. 2002). Gammarids are prominent elements of the limnofauna of the northern hemisphere and widely abundant in streams. Because their habitats are potentially influenced by estrogen hormone residues introduced by way of sewage treatment plant effluents, a disruption of sex ratios and reproductive cycles, or the regulation of the endocrine system, would be crucial for the fate of established natural populations. However, population size and structure may also vary considerably under natural conditions, and it is unclear whether other environmental parameters of nonchemical character could even have a larger influence on reproductive parameters in *Gammarus* spp. than potential estrogens.

To investigate the impact of both natural environmental variability and artificially introduced household wastewaters, we used the following study design. We addressed spatial variability by choosing two small streams, one in Southwest Germany and the other in Eastern Germany; we addressed seasonal aspects by sampling in spring and autumn; and we also considered variability of two sequential years. In contrast, the rather continuous parameter in this study was the influence of each sewage plant on both streams in whose effluents potential endocrine disruptors were present. Using upstream and downstream sampling sites at both streams, the influence of potential sources of endocrine-disrupting chemicals was tested under field conditions. At the beginning of our study, no information on the mechanisms of the endocrine action of xenobiotics in *Gammarus* spp. was available. Therefore, the selection of

end points, presumably indicating effects on the reproductive system, was biased. Because Tan-Fermin and Pudadera (1989) have described the female maturation cycle in crustaceans, it was reasonable to select histologic end points of oocyte maturation as measures of reproductive cycle integrity. At the biochemical level, Schirling et al. (2004) found a strong covariation of the level of the protein hsp90 with the reproductive cycle. In early stages of oocyte development, the hsp90 level of individuals was lower than in specimens with mature eggs by a factor of three. In vertebrates, it is known that hsp90 is of crucial importance for steroid receptor interactions and modulates sex hormone signal transduction (Pratt and Toft 1997). The existence of an equally complex system has not been proven for invertebrates. However, the recent discovery of estrogen receptors in invertebrate taxa, both in the deuterostome and protostome clade (de Waal et al. 1982; di Cosmo et al. 2002) indicate that steroid-binding proteins and therefore mechanisms associated with the signal transduction process are phylogenetically very old (Thornton et al. 2003). We selected hsp90 as a potential biochemical end point, particular in view of possible uncoupling effects, although it is well known that hsp90, like all stress proteins, also responds to stressors that do not target the endocrine system. As a measure of such non–endocrine-disruptor stressor effects, we therefore included the well-established general stress marker hsp70 in our study, predominantly as an ‘‘unspecific stress effect control’’ for hsp90.

The aim of this study was to answer (1) whether we can trace possible endocrine effects in gammarids in a highly variable environment; (2) whether biomarker responses occur predominantly downstream of sewage plant discharges; and (3) whether variability in the selected biomarkers can be attributed to the influence of endocrine-disrupting potentials or, alternatively, to environmental parameters varying in space and time.

MATERIAL AND METHODS

Field exposure

In 2000 and 2001, adult male and female gammarids (*Gammarus fossarum* [Koch 1835]) from two streams were collected for this study. One of the streams, the Lockwitzbach near Dresden, East Germany, has a lower impact of sewage treatment plant effluent, whereas the Körsch, near Stuttgart, Southwest Germany, comprises a mixture of pollutants caused by intensive agriculture and a series of sewage treatment plants (Adam et al. 2001). To examine

environmental effects on *G. fossarum* upstream and downstream of the effluent of the most upstream sewage treatment plant, two sampling sites were established in each stream. The four sites were referred to as Lu (Lockwitzbach upstream), Ld (Lockwitzbach downstream), Ku (Körsch upstream), and Kd (Körsch downstream). At both streams, the upstream and downstream sites were approximately 6 km apart. The content of all four sampling sites, plus the respective waste water effluent, were characterized by chemical analyses for a series of steroids, phenols, phthalates, pesticides, and their metabolites every month between April and October 2001 (results presented in Jungmann et al. 2004b). Furthermore, limnochemical parameters were analyzed in parallel to the animal samplings, and, additionally, every 4 weeks between April and October in both 2000 and 2001 (Table 1). In both years of the investigation, two samplings took place, one in April and one toward the end of the reproductive period of *G. fossarum* in October. At each site, gammarids were collected by kick sampling and their body length measured and separated into two groups: medium (M) size ($6 \leq x \leq 9$ mm) and large (L) size ($x > 9$ mm). The number of replicates differed according to site, sampling time, and investigated parameter because of the abundance of gammarids, percentage of male animals in the sample, etc. (Table 2).

Stress protein analysis

For the stress protein (hsp90 and hsp70) analysis, the gammarids were shock frozen in liquid nitrogen on-site. The animals were individually homogenized in extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM Hepes, and 2% protease inhibitor [Sigma P8340, pH 7.5], the volume of which was adjusted to the individual's body weight and the homogenate subsequently centrifuged (for 12 minutes at 20,000 g at 4°C). Total protein concentration in the supernatant was determined according to the method of Bradford (1976). Constant protein weights (40 µg total protein per lane) were subjected to sodium dodecyl sulphate-polyacrylamide gel (12% acrylamid-bisacrylamid) for 20 minutes at 80 V and for 120 minutes at 120 V in duplicate (samples for hsp90 and hsp70 analysis were run on separate gels). Protein was transferred to nitrocellulose by way of semidry blotting, and the filter was blocked for 2 hours in 50% horse serum in Tris-buffered saline (TBS; 50 mM Tris and 150 mM NaCl, pH 7.5). After washing in TBS, monoclonal antibody (mouse antihuman hsp70; Dianova, Hamburg, Germany, dilution 1:5,000 in 10% horse serum/TBS or mouse anti-water mold hsp90; StressGen Victoria, Canada, dilution 1:800) was added and incubated at room temperature overnight. After repeated washing in TBS for 5 minutes, the nitrocellulose filters

were incubated in the second antibody (peroxidase-conjugated goat anti-mouse immunoglobulin G; Dianova, dilution 1:1,000 in 10% horse serum/TBS) at room temperature for 2 hours. After repeated washing in TBS for 5 minutes, the antibody complex was detected by 1 mM 4-chloro(1)naphtol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol. The grey scale values of the Western blot protein bands were quantified using a densitometric image analysis system (Herolab E.A.S.Y., Germany) and related to an hsp90 or, respectively, an hsp70 standard run in parallel on each gel.

In the primary experimental set-up, only hsp90 measurements were included, which explains the lack of data on hsp70 for the first sampling. To assess the general stress status in the gammarids, however, we decided also to include hsp70 analysis from fall 2000 onward.

Histological analysis

On site, the gammarids were decapitated and fixed in 2% glutardialdehyde and dissolved in 0.005 M cacodylate buffer. Before embedding, the gammarid samples were decalcified in 5% trichloroacetic acid for 2 days. The samples were dehydrated in a graded series of ethanol and finally embedded in Technovit (Heraeus Kulzer, Germany). Then the animals were sectioned as follows: per individual, 8 series each of 16 sagittal sections (4 µm thickness each) were cut on a Reichert Jung 2050 microtome. The arrangements of the 8-section series depended on the individual width of each specimen, thus assuring a complete overview of the histology of each individual. Sections were stained with Methylene blue-Azzur II according to Richardson et al. (1960), and examined using a light microscope (Zeiss, Axioscop 2).

The female animals were classified based on the maturity status of their gonads. This was accomplished by examining the maturity of the oocytes. In this study, five different stages of oocytes were distinguished on the basis of the work of Tan-Fermin and Pudadera (1989) (Fig. 1): (1) previtellogenic stage (P) = small basophilic cells with a large nucleus, but lacking vesicles; (2) early vitellogenic stage (E) = small basophilic cells with few vesicles in the cytoplasm and occurrence of perinuclear heterochromatin; (3) late vitellogenic stage (L) = larger, cubic cells with large vacuoles of lipids or proteins, and follicle cells surround the outer membrane of these oocytes; (4) mature stage (M) = last stage of the oocytes before being released to the marsupium; characteristic egg membrane surrounds the cell, and the ellipsoidal nucleus is located next to the membrane; and (5) spent stage (S) = only oocytes which are not released into the marsupium belong to this stage; they become atretic and undergo lysis. In addition, a sixth ‘‘stage’’ of oocyte development at the beginning of the next reproductive

cycle was distinguished: intermediate stage (SP): most cells are in spent stage; in addition, previtellogenic oocytes of the next reproductive cycle have already occurred.

Tab.1: Physico-chemical parameters at the different sampling sites in 2000 and 2001

Parameter		Lu	Ld	Ku	Kd
Oxygen	Median	10,1	9,8	9,3	9,5
[mgl^{-1}]	25-75%	9,7/10,9	9,3/10,5	8,8/10,6	9,1/10,3
	min/max	9,0/11,8	8,2/12,0	6,9/13,7	7,9/11,8
pH-value	Median	7,8	7,7	8,2	8,2
	25-75%	7,6/7,9	7,4/7,8	8,1/8,3	8,1/8,3
	min/max	6,4/8,0	0,0/8,5	7,7/8,9	8,0/8,6
temperature	Median	12,6	13,1	13,0	14,8
[°C]	25-75%	11,0/14,4	11,6/15,0	11,8/14,4	13,3/16,0
	min/max	6,0/16,1	7,0/15,8	8,0/16,3	10,6/17,4
Conductivity	Median	355	589	917	838
[μScm^{-1}]	25-75%	330/374	502/629	880/947	800/909
	min/max	267/467	435/908	711/1725	533/960
Nitrate-N	Median	7,0	9,8	1,9	6,9
[mgl^{-1}]	25-75%	6,0/8,6	7,3/11,1	1,5/2,3	4,9/9,4
	min/max	4,1/11,7	4,6/14,2	1,1/8,4	2,8/11,1
Nitrite-N	Median	0,01	0,03	0,01	0,02
[mgl^{-1}]	25-75%	0,01/0,03	0,01/0,05	0,01/0,02	0,01/0,04
	min/max	0,00/0,05	0,01/0,13	0,00/0,15	0,01/0,09
Ammonium-N	Median	0,02	0,07	0,04	0,02
[mgl^{-1}]	25-75%	0,02/0,10	0,03/0,10	0,01/0,07	0,01/0,09
	min/max	0,00/0,10	0,00/0,75	0,00/0,20	0,00/0,34
Ammonia-N	Median	0,00	0,00	0,00	0,00
[mgl^{-1}]	25-75%	0,00/0,00	0,00/0,00	0,00/0,00	0,00/0,00
	min/max	0,00/0,00	0,00/0,20	0,00/0,01	0,00/0,01
SRP-P	Median	0,20	0,31	0,52	0,94
[mgl^{-1}]	25-75%	0,19/0,23	0,26/0,40	0,21/0,90	0,45/2,13
	min/max	0,10/0,30	0,15/0,79	0,11/11,3	0,34/3,9
Silicon	Median	5,0	6,2	nd	nd
[mgl^{-1}]	25-75%	4,4/5,3	5,3/6,6	nd/nd	nd/nd
	min/max	3,0/6,2	4,7/7,8	nd/nd	nd/nd
total H	Median	9	15	13	11
[°dH]	25-75%	8/11	13/16	11/15	8/14
	min/max	4/16	7/19	8/18	5/17
carbon H	Median	4	8	12	11
[°dH]	25-75%	3/4	7/10	10/14	8/12
	min/max	1/9	5/13	8/18	8/14
non carbon H	Median	5	6	1	1
[°dH]	25-75%	4/7	5/7	0/2	-2/3
	min/max	-5/12	-5/11	-5/3	-4/5

Lu: Lockwitbach upstream; Ld: Lockwitzbach downstream; Ku: Körsch upstream; Kd: Körsch downstream; SRP: soluble reactive phosphate; H: hardness.

The furthest developed stage of intact (i.e., nonatretic) oocytes was considered to define the developmental stage of the respective individual. Thus, each individual was classified as belonging to one of the stages previously described: SP, P, E, L, M, or S. The developmental

stage of all female gammarids at a given time, location, and size class was expressed by a maturity index (mi) according to the following equation: $mi = (x_{SP} + 2x_P + 3x_E + 4x_L + 5x_M + 6x_S) * 10^{-2}$, where x_{SP}, x_P, x_E, x_L, x_M, and x_S refer to the percentages of the individuals in the respective stages SP, P, E, L, M, and S in the entire sample.

Additionally, both intact and atretic oocytes in the early vitellogenic stage and in the late vitellogenic stage were recorded. To distinguish the types of oocytes from the developmental stage of the entire individual, early vitellogenic oocytes were abbreviated EVO, and late vitellogenic oocytes were abbreviated LVO. The ratio between atretic and intact oocytes of the EVO and LVO type was calculated for each individual displaying oocytes of at least one of these two types. Furthermore, the area of intact oocytes of both the EVO as well as the LVO type in the section was determined by morphometric software (Openlab 3.0, Improvision) and a mean calculated for every individual displaying one of theses types.

Table 2. Number of replicates per sampling for histologic and biochemical investigations

	Histology				hsp90 / hsp70			
Month and Year	Lu	Ld	Ku	Kd	Lu	Ld	Ku	Kd
M size								
Apr 2000	18	17	16	9	8 / 0	5 / 0	8 / 0	9 / 0
Oct 2000	9	9	6	6	10 / 8	8 / 7	8 / 5	10 / 9
Apr 2001	10	18	11	13	10 / 10	10 / 15	10 / 7	11 / 14
Oct 2001	17	17	8	9	18 / 12	19 / 12	9 / 10	10/ 10
L size								
Apr 2000	9	13	7	0	9 / 0	6 / 0	6 / 0	9 / 0
Oct 2000	20	2	2	3	10 / 10	10 / 10	9 / 8	8 / 7
Apr 2001	10	16	10	6	10 / 10	9 / 12	8 / 1	2 / 4
Oct 2001	6	9	3	1	8 / 0	8 / 4	18 / 20	11 / 20

Kd = Körsch downstream; Ku = Körsch upstream; Lu = Lockwitzbach upstream; Ld = Lockwitzbach downstream.

Statistical analysis

For statistical analysis we used the JMP 4.0 (SAS) software. Normally distributed data (checked by Shapiro-Wilk's test, $p \leq 0.05$) were tested for significance using Student's t test, whereas data with nonnormal distribution underwent Kruskal Wallis analysis of variance (ANOVA). The α-level for significant differences was set at $p \leq 0.05$. Pairwise linear

correlation analysis used the calculation of the Pearson product-moment correlations for each pair of variables ($p \leq 0.05$). Only the variables (exposure parameters) with significant linear correlation to target variables (biologic responses) were integrated in multiple regression models to explain the maximum percentage of the variability of these biologic responses. Considering the limited number of data sets and the biologic significance of extreme values, cluster analysis was performed with hierarchical clustering using Ward's minimum variance method (Milligan 1980).

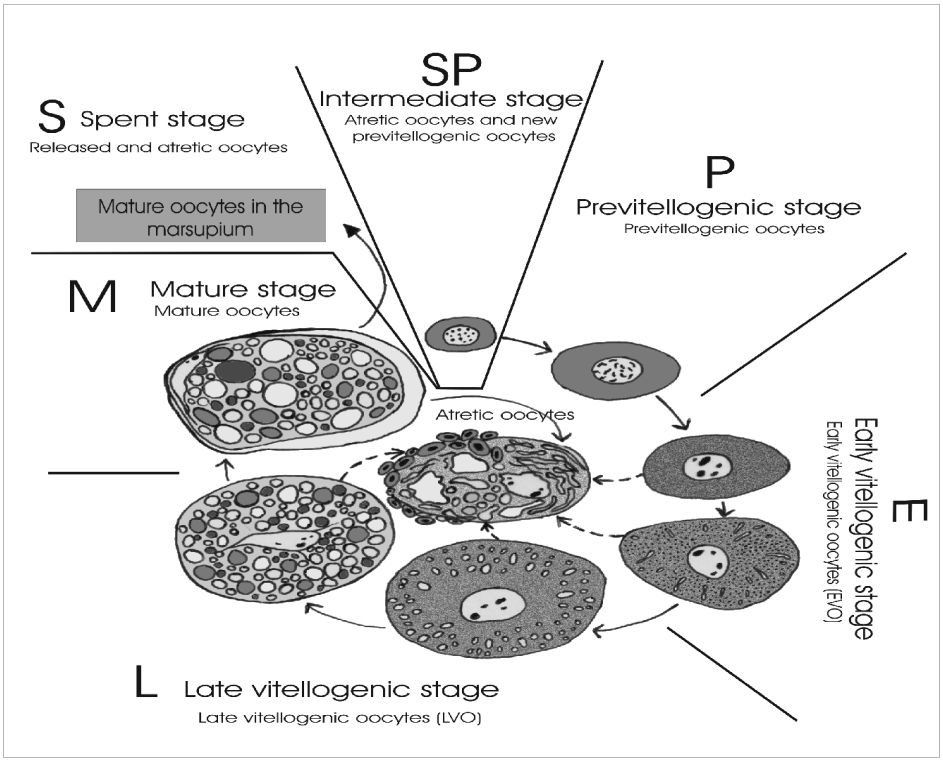

Fig. 1. Different oocyte stages according to Tan-Fermin and Pudadera (1989). Explanation is in the text.

RESULTS

Differences in stress protein and histology between the upstream and downstream sites

For hsp70 and hsp90, no uniform pattern was observed at the Lockwitzbach site that would give any information about the influence of the discharger. The same was true for the M-size gammarids, independent of their origin, except for a significant decrease of the hsp90 level at the Körsch downstream site in spring 2001. The L-size gammarids from Körsch, however,

revealed both hsp90 and hsp70 to be tentatively or significantly decreased at Kd comparison with Ku in spring and autumn of both years (Fig. 2).

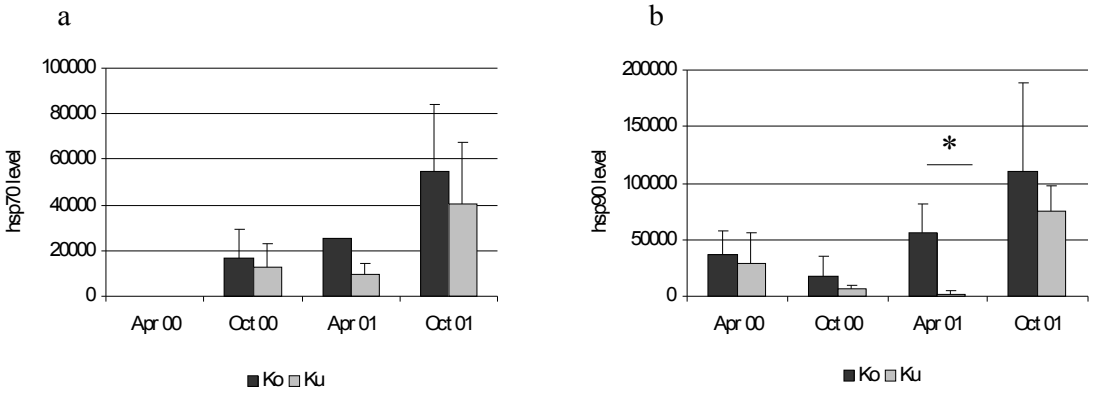

Fig. 2. (a) Mean hsp70 levels (= optical volume = number of pixels x average densitometric intensity of Western blot bands) and SD of L-size gammarids on Ku and Kd sites. (b) Mean hsp90 level (grey scale values) and SD of identical individuals. Kd = Körsch downstream; Ku = Körsch upstream.

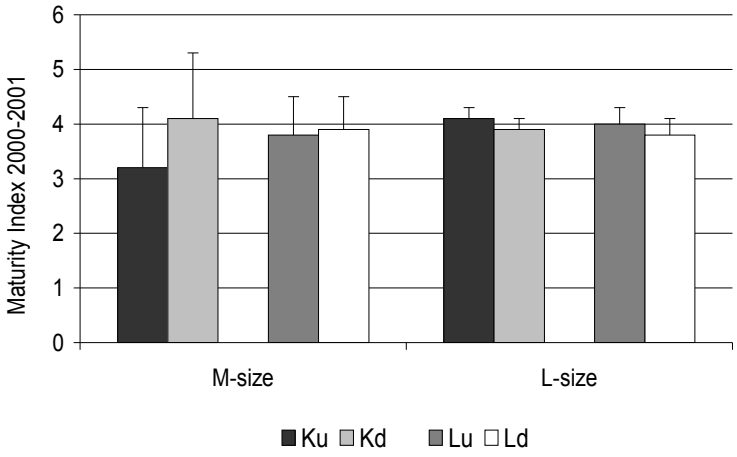

Fig. 3. Maturity index of M- and L-size gammarids at the four sampling sites: Ku, Kd, Lu, and Ld (means for four samplings during 2000 and 2001 each, and SD). Kd = Körsch downstream; Ku = Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitzbach upstream.

For the histologic parameters, tentative differences between the sampling sites occurred only at the Körschsite. Contrary to the heat shock proteins, the differences in histologic end points were visible only in M-size gammarids (Fig. 3). In 2000, the samplings in spring and fall revealed a higher maturity index (indicating the animals to be further along in their development) in M-size gammarids at Kd compared with Ku. In 2001, this trend hardly was visible (Fig. 4).

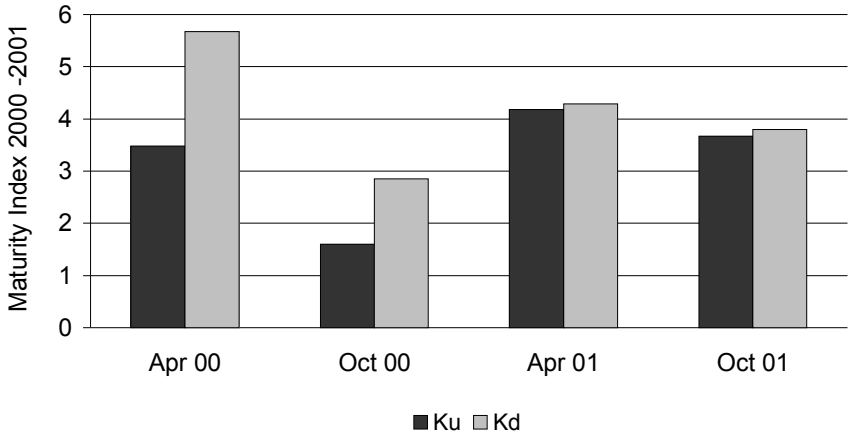

Fig. 4. Maturity index of M-size gammarids at the two sampling sites, Ku and Kd, in 2000 and 2001. Kd = Körsch downstream; Ku = Körsch upstream.

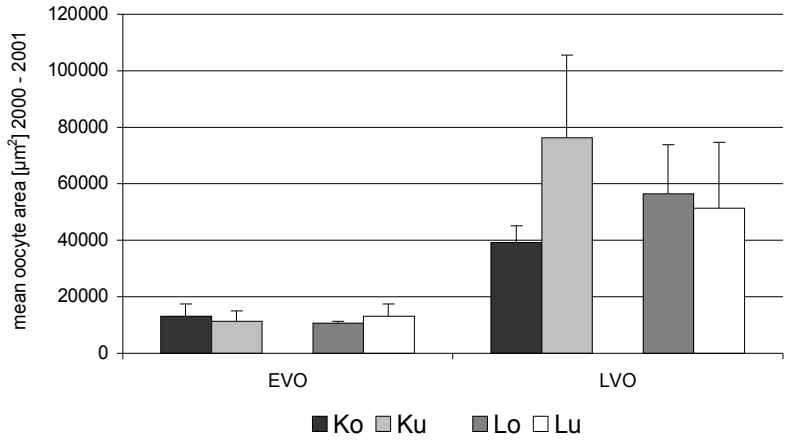

Fig. 5. Oocyte area for M-size gammarids. Means (of the four samplings in 2000 and 2001) and SDs at the four sampling sites: Ku, Kd, Lu, and Ld. Kd = Körsch downstream; Ku = Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitzbach upstream.

Because the investigated parameters maturity, oocyte area, and atresia are not entirely independent from one another, the area and atresia of LVOs largely supported the data found in the maturity analysis. The areas of LVO in particular were on average larger downstream that upstream from the sewage plant effluent at the Körsch river site (significant at $p \leq 0.05$ for April 2001) in M-size animals, whereas EVOs did not show any differences (Figs. 5 and 6). Furthermore, the level of LVO atresia in three of four samplings was higher at Kd compared with Ku, although the differences were not significant. In general, atresia at Kd was significantly higher than at Ld (Fig. 7).

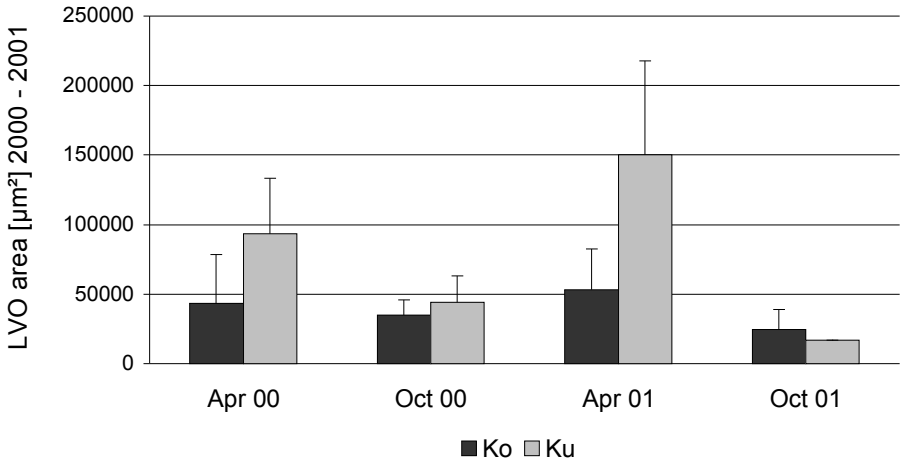

Fig. 6. Area of LVO in M-size gammarids at the two sampling sites, Ku and Kd, in 2000 and 2001. Means and SDs. Kd = Körsch downstream; Ku = Körsch upstream; LVO = late vitellogenic oocytes.

Concomitantly, correlation analysis showed a positive correlation of maturity index and LVO cell area ($p = 0.002$), maturity index and LVO atresia ($p = 0.002$), and LVO cell area and LVO atresia ($p = 0.004$) in M-size gammarids. Stress protein hsp70 and hsp90 levels were found to positively correlate in L-size gammarids ($p = 0.026$) and, tentatively, M-size specimens ($p = 0.055$). The only significance in a comparison of stress proteins and histologic parameters was found in a negative correlation of hsp70 and LVO cell area in M-size gammarids ($p = 0.015$).

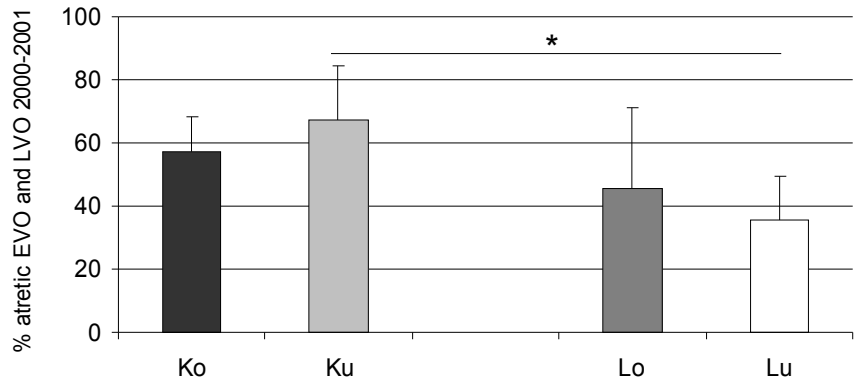

Fig. 7. Percentage of atretic oocytes (means of atresia in both LVO and EVO for all four samplings during 2000 and 2001 and SDs) in M-size gammarids at the four sampling sites: Ku, Kd, Lu, and Ld. EVO = early vitellogenic oocytes; Kd = Körsch downstream; Ku = Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitzbach upstream.

Cluster analysis of own and literature data

Using the data set obtained for hsp90, maturity index, EVO and LVO cell area, and EVO and LVO atresia for both M- and L-size classes, cluster analysis revealed four main clusters of respectively similar parameter patterns (hsp70 could not be included because the data set for 2000 was incomplete). These four clusters were particularly characterized by the sampling events in spring 2000, fall 2000, spring 2001, and fall 2001 (Fig. 8). In three of the four main clusters, the analysis additionally separated the two investigated streams from one another. A similar pattern of variables in locations either upstream or downstream from a sewage treatment plant, respectively, could not be found. The separation of this dataset into two subsets, according to the site classes M or L, resulted in different patterns in these two size classes. The subset for L-size animals did not show any clustering according to the variables sampling time, stream identity, or upstream or downstream location. In contrast, the data subset obtained for the M-size class revealed predominantly the importance of the parameter, season, which characterized the two main cluster in this analysis (Fig. 9).

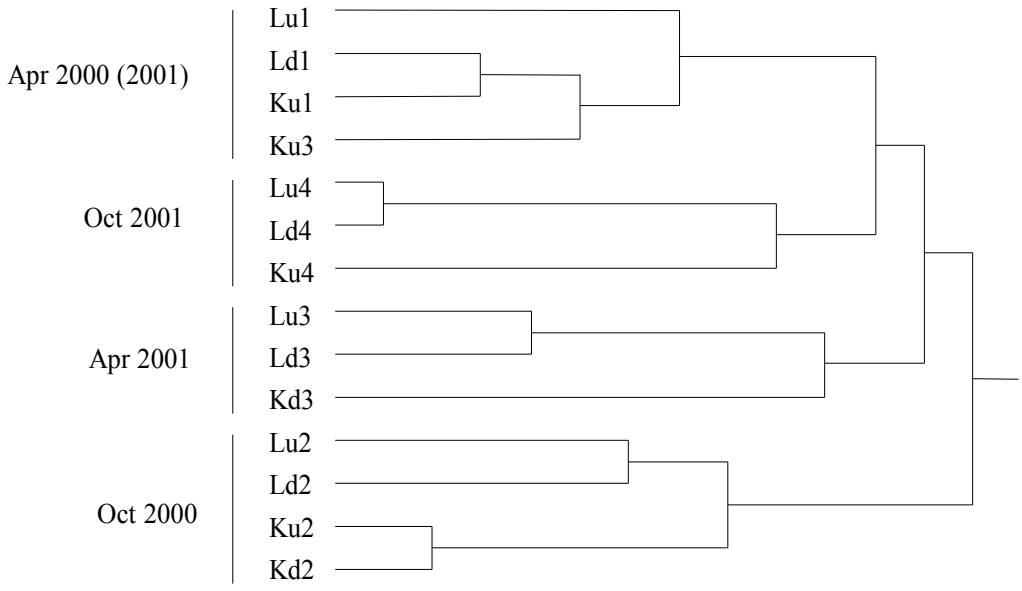

Fig. 8. Dendrogram according to the cluster analysis of the data set for maturity index, area LVO, area EVO, atresia LVO, atresia EVO, and hsp90 in both M- and L-size gammarids. 1 = April 2000; 2 = October 2000; 3 = April 2001; 4 = October 2001. Mean clusters are indicated by vertical lines. EVO = early vitellogenic oocytes; Kd = Körsch downstream; Ku = Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitzbach upstream; LVO = late vitellogenic oocytes.

The exposure situation at the four locations had been characterized regarding limnochemical data (Jungmann et al. 2004a) and potential endocrine disruptors (Jungmann et al. 2004b). Using the data obtained for the limnochemical parameters listed in Table 1 at the times of gammarid sampling, cluster analysis separated three main clusters: one fall cluster (comprising data sets for autumn 2000 and 2001) and two separated spring clusters, one for spring 2000 and the other for spring 2001. Thus, the pattern of the entire set of limnochemical parameters was season rather than site specific. The same was true for the total set of potential endocrine disruptors (Jungmann et al. 2004b) present in the streams at the times of gammarid sampling. The main clusters separated between the time of sampling while spatial aspects were of minor importance and restricted to the identity of streams but not to the location of the sites up- or downstream the sewage treatment plants.

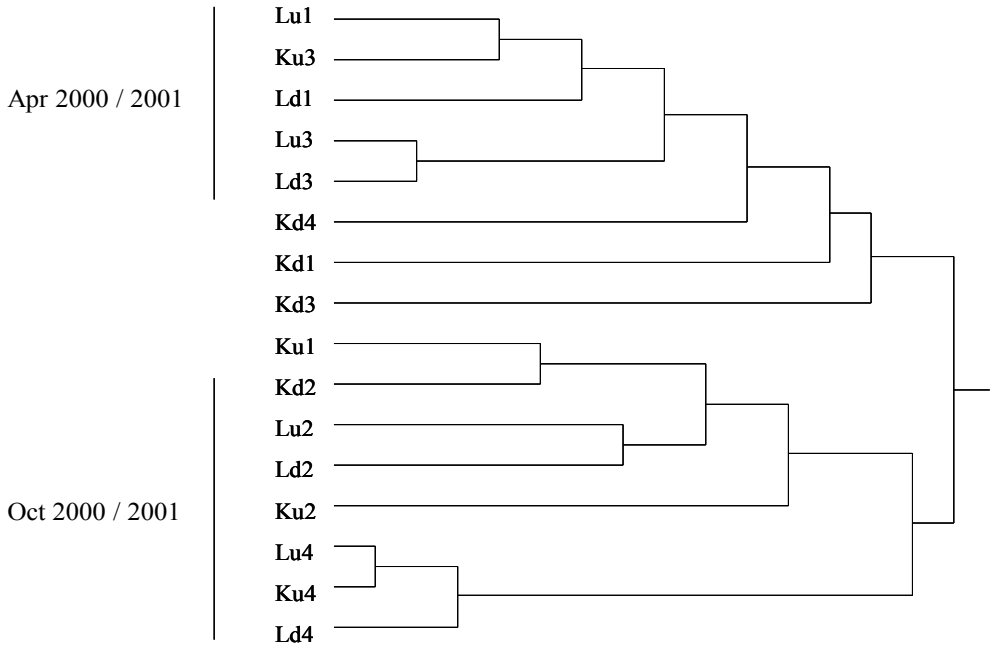

Fig. 9. Dendrogram according to the cluster analysis of the dataset for maturity index, area LVO, atresia LVO, and hsp90 in M-size gammarids. 1 = April 2000; 2 = October 2000; 3 = April 2001; 4 = October 2001. Mean clusters are indicated by vertical lines. Kd = Körsch downstream; Ku = Körsch upstream; Ld = Lockwitzbach downstream; Lu = Lockwitzbach upstream; LVO = late vitellogenic oocytes.

Correlation analysis and multiple regression modelling

To test not only for patterns of multiple variables but to detect exposure parameters of potential biologic relevance, correlation analysis was performed for a number of biologic parameters that were found to differ between upstream and downstream sites and the parameters of exposure (Table 1; Jungmann et al. 2004b). The maturation index of M-size gammarids was positively correlated with oxygen concentration ($p = 0.016$), oxygen saturation ($p = 0.010$), total hardness ($p = 0.043$), concentration of DDT ($p = 0.049$, $n = 4$), and particularly with concentration of di-n-octyl phthalate (DnOP) ($p = 0.0002$). A multiple regression model incorporating only DnOP and DDT explained 93.5% of total maturation index (M-size) variability with a significant ($p = 0.016$) contribution of DnOP to the model. Although the parameter LVO area (M-size) did not show any correlation with single exposure parameters, the level of atresia in LVO (M-size) was correlated positively with DnOP ($p = 0.020$) and DDT ($p = 0.004$, $n = 4$) and tentatively positively correlated with the concentration of dimethyl phthalate ($p = 0.055$). A multiple regression model using DnOP and DDT explained 78.6 % of LVO atresia (M-size) variability.

Corroborating the observed decrease of the hsp90 level downstream from the sewage plant discharge at Körschriver, hsp90 showed mainly negative correlation with exposure parameters. In M-size gammarids, the level of this stress protein did not correlate with any limnochemical parameter, but it did correlate exclusively and negatively with DnOP ($p = 0.032$) and DDT ($p = 0.026$, $n = 4$) and positively with the concentration of dibutyl phthalate ($p = 0.018$). Of hsp90 (M-size) variability, 63.2% could be explained by a multiple regression model using DnOP and DDT. In L-size animals, the only correlation of the hsp90 level was a negative relationship with the concentration of DDD ($p = 0.011$, $n = 4$, explaining 69.0 % of hsp90 [L-size] variability).

DISCUSSION

Although different size classes of *G. fossarum* individuals were investigated separately, a high variability in the biologic parameters was recorded, which prevented significant differences in most cases. This variability results at least partially from differences in the developmental status of individuals within a distinct size class (Pöckl and Humpesch 1990). Nevertheless, effects of the respective exposure situations on gonad development and hsp90 level, both parameters related to the endocrine system, could be found in the Körsch river: differences in

maturity of oocytes, larger late vitellogenic oocytes, increasing atresia, and decreasing hsp90 levels occurring downstream from the discharge of a sewage treatment plant. At the Lockwitzbach site, these effects were not found. Indeed, the main difference between the two rivers was in the amount of discharged water, which is much higher at the Körsch than the Lockwitzbach site. As a consequence, a higher estrogenic potential introduced by the sewage plant effluents was measured at the Körsch site (Jungmann et al. 2004b). Even though an influence of sewage plant effluent on the investigated biologic parameters is likely at least for the situation at the Körsch site, solely for this reason the effects must not necessarily be related to analyzed xenohormones because a number of other pollutants, e.g., PAHs, PCBs, and pesticides, are introduced into this river as well (Honnen et al. 2001). Nevertheless, there is evidence for the interaction of external parameters with the internal reproductive cycle in *G. fossarum*.

At the cellular and individual level, biologically linked parameters such as maturity stage, the size of more developed oocytes and the number of atretic LVO cells (symbolizing leftover oocytes that have not been released into the brood pouch) correlated in a positive way. In contrast to noncontaminated laboratory experiments (Schirling et al. 2004), however, the field situation resulted in a disruption of the endogenous hsp90 cycle (initially a positive relationship with increasing maturity) and the female reproductive cycle (negative correlation instead). Furthermore, in the investigated field sites, hsp90 and hsp70 levels were found to correlate rather in a positive way (in contrast to the negative correlation under controlled laboratory conditions), presumably representing hsp90 as acting as a stress protein along with hsp70. Although it is known that heavy pathologic impact usually affects biochemical processes, the uncoupling of oocyte maturation and the hsp90 cycle in this case cannot be interpreted as a result of histopathology. Histology did not reveal pathologic changes in the gonads of *G. fossarum*. Furthermore, in this study the abundance of *G. fossarum* was found to be highest at the Kd sampling site (Ladewig and Nagel 2004), possibly because of rich nutritional supply. If the decrease of hsp70 and hsp90 had been related to an irreversible cessation of protein synthesis, such an injured population would hardly be stable. The increase of atresia in LVO cells also must be interpreted as a result of resorption of oocytes that have not been released into the marsupium (because of the spatial limitations of this brood pouch) rather than as resulting from histopathologic impact (as described by Yashodhara 2002). Otherwise, EVO cells or other earlier oocyte stages would have exhibited increased atresia as well. Therefore, we assume that other potential endocrine disruptors (which have not been

analyzed) and abiotic parameters interact with regulation processes in a nondestructive way, thus resulting in a high temporal and spatial variability of parameters related to the reproductive cycle.

Indeed, as shown by cluster analysis, the pattern of exposure parameters, represented by the limnochemical and analytic chemistry data, varied predominantly temporally. Therefore, the influence of the wastewater discharge was only visible for single parameters but not for the entire pattern (thus ‘‘disguising’’ the possible importance of single compounds to some extent). Under these circumstances, it is reasonable that the general pattern of biologic responses depended predominantly on temporal aspects such as the season or the sampling time. Although the selection of investigated biologic parameters took place particularly to trace ‘‘endocrine’’ effects, a number of these parameters consequently must be regarded as to have been considerably influenced by those environmental factors that do not specifically target the endocrine system. However, correlations of single parameters in specific size classes - such as the stage of maturity, the size of LVO cells and the ratio of LVO atresia in animals of 6-9 mm in length, as well as the hsp90 level in animals >9 mm with single-exposure parameters - indicate the importance of potential xenohormones for *G. fossarum* development. A number of observations support this interpretation: As shown by Watts et al. (2002), the genus *Gammarus spp*. is per se susceptible to estrogenic compounds. Responses of the mentioned cytologic and biochemical parameters were only observed at the Kd sampling site, characterized by an increased estrogenic potential. In a parallel study, Ladewig and Nagel (2004) showed demographic parameters in *G. fossarum* to be altered at the Kd sampling site. We found a significant correlation of different mentioned cellular and molecular markers always with the same compounds (DnOP and DDT or its metabolite, DDD), and models showed a high potential of these exposure variables to explain the biologic responses. Correlation with other abiotic variables, such as temperature, pH, phosphorous and nitrogen compounds, etc., was lacking, thus indicating the minor importance of these parameters for the mentioned biotic responses. In a laboratory experiment with a single potential estrogenic compound, bisphenol A, the identical tendency of *G. fossarum* to respond with a higher maturation index, larger LVO cells, a higher rate of LVO atresia, and a decreasing hsp90 level (Köhler and Triebskorn 2004; Schirling and Köhler 2004) mirrored the situation at the downstream location at the Körschsite.

Because mechanistic studies on the interaction of potential xenohormones with the endocrine system in *Gammarus spp*. are still lacking, these observations give presumptive

evidence only. The fact that one size class of gammarids showed an effect on one criterion and a different size class responded with another may well reflect the complexity of the pollution situations examined and the potential for interactions at the different sampling conditions. Clearly, more single-pollutant laboratory studies are needed in which specific correlation with response criteria can be made. However, in view of the precautionary principle, the mentioned single biologic parameters that were found to be influenced in this study can be recommended as indicators of possible influence of potential xenohormones on *G. fossarum*. Considering the technical aspects, this certainly is advantageous for biotests aiming at the detection of potential endocrine effects on crustaceans. As shown in this study, the field situation, however, is much more complex, and effects on single cellular or biochemical parameters may be hidden or even compensate for seasonal cycles of gonad development

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Kapitel 3

BISPHENOL A IN ARTIFICIAL INDOOR STREAMS: II. STRESS RESPONSE AND GONAD HISTOLOGY IN *GAMMARUS FOSSARUM* (AMPHIPODA)

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ABSTRACT

The effects of the worldwide distributed chemical bisphenol A (BPA) on the endocrine system of vertebrates have been demonstrated in several studies. Here, we report on the impact of bisphenol A (0; 5; 50 and 500µg/L nominally, deduced effective concentrations 0; 0.24; 2.4; and 24.1µg/L respectively, all at 15°C) on the70 kD stress protein family (hsp70), the 90 kD stress protein family (hsp90), and gonad histology of the crustacean *Gammarus fossarum* exposed in artificial indoor streams. The animals were exposed for a maximum of 103 d and samples were taken at the beginning and at days 34, 69 and 103 of the experiment. Exposure to BPA resulted in accelerated maturation of oocytes in females and in a decline in the number and size of early vitellogenic oocytes. The level of hsp90, which plays a pivotal role in vertebrate sex steroid signal transduction, was significantly reduced by BPA.

In all five streams, measured parameters did not indicate any captivity stress for a period of 69 days. Beyond this time, the mortality rate and proteotoxic effects, the latter measured by hsp70 expression, were found to be elevated

Keywords: bisphenol A, *Gammarus fossarum*, stress proteins, gonad histology, artificial indoor stream

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Ecotoxicology (in press)

INTRODUCTION

Bisphenol A is a potential endocrine disruptor of considerable environmental significance because it is potent and used in large quantities (Colburn, 2002; Gullette and Gunderson, 2001; McLachlen, 2001). Its diverse effects on the endocrine system have been reported (e.g. European Chemical Bureau, 2003; Stocker et al., 2003; Jobling et al., 2002; Schönfelder et al., 2002; Staples et al., 1998). World wide, approximately 2.5 million tonnes BPA per year are produced (Staples et al., 2002). It is a key building block of polycarbonates and can be found in many products like epoxy resin, dental sealants and composites, as well as in coatings of metal food and beverage cans. So far, only a few studies have reported effects of BPA on invertebrates (Duft et al., 2003; Segner et al., 2003; Pascoe et al., 2002; Jobling et al., 2002; Watts et al., 2001a) and the mode of action in these organisms remains still unknown.

A common problem, not only in studies on endocrine disrupting chemicals, is to transfer results from laboratory experiments into natural ecosystems or vice versa. To bridge this gap between laboratory and field, "semi-natural" exposure systems, such as flow-through bypass systems (Triebskorn et al., 2001) or artificial indoor streams (Jungmann et al., 2001), have been established in recent years. Using a set of artificial indoor streams in this study, we had the advantage of continuously controlled conditions regarding physical and limnochemical parameters as well as the exposure to BPA. To prove proper conditions within such artificial systems, it was necessary to check the exposed gammarids for their general stress by measurements of their 70kDa stress protein (hsp70) level.

Bisphenol A is introduced into aquatic ecosystems via the effluents of industrial and municipal sewage treatment plants. Since gammarids are among the most common invertebrates in streams and rivers, we investigated possible effects of BPA in the amphipod *Gammarus fossarum* (Koch 1835), a widely abundant species in European streams. *G. fossarum* is a keystone species and a representative for other invertebrates of the lotic communities because of its function as shredder and its importance for the food chain. As previous studies indicated, BPA is able to affect reproductive and developmental processes in invertebrates like the number of eggs produced by *Potamopyrgus antipodarum* (Duft et al., 2003) or the emergence time and percentage of adults in *Chironomus riparius* (Watts et al., 2001b). As well, our endpoints for the investigations were selected according to their association with the reproductive system, and included biochemical as well as histological parameters.

We used the correct maturation of oocytes and the occurrence of atresia in the ovary as histological markers to detect variations in the reproductive cycle of gammarids. These biomarkers were selected based on the work of Tan Fermin and Pudadera (1989), who described the female maturation cycle in crustaceans, and a strong co-variation of the level of the stress protein hsp90 with the reproductive cycle in *G. fossarum* shown by Schirling et al., 2004. In that study, the hsp90 level of individuals in early stages of the oocyte development was a factor of 3 lower than in specimens with mature eggs. In vertebrates, hsp90 is of crucial importance for steroid receptor interactions and modulates sex hormone signal transduction (Pratt and Toft, 1997). For invertebrates an equally complex system has not been proven yet. However, the recent discovery of estrogen receptors in invertebrate taxa, both in the deuterostome and protostome clades (de Waal et al., 1982; di Cosmo et al., 2002) indicates that steroid-binding proteins and, therefore, mechanisms associated with the signal transduction process are phylogenetically very old (Thornton et al., 2003). Therefore in the present study, hsp90 was selected as a potential biomarker, particular in view to possible uncoupling effects. Like all stress proteins, hsp90 also responds to stress factors, which do not directly target the endocrine system. Therefore, we included the well-established general stress marker hsp70 in our study, predominantly as an 'unspecific stress effect control' for hsp90.

Our major objectives was to determine whether BPA induces changes in the hsp90 levels or alterations in the reproductive cycle of *G. fossarum*, and whether these possible effects can be separated from effects caused by unspecific stress.

MATERIAL AND METHODS

Experimental design

The studies reported here were performed between May 2002 and September 2002 at Dresden University of Technology, Germany. The artificial indoor stream system was located in a greenhouse, the five identical streams were 3.70 m long and 50 cm wide (Jungmann et al., 2001). Twenty-one days before the first application of bisphenol A (BPA), and the start of the experiment, the bottom of each stream was covered with gravel of different size (2–8 mm [46%], 8–16 mm [31%], 16–32 mm [23%]). Each end of the streams had a water reservoir which was separated by a fine mesh sieve to prevent loss of organisms from the stream. The

streams were filled with tap water and micronutrients and salts were added according to Borgmann (1996). The water level was between 9 to 12 cm which corresponded to a volume of 471 to 502 L per stream. In each stream the water was circulated by a pump with a flow velocity of the water of 0.2 m sec^{-1}. For the experiment, five artificial indoor streams were run under the following conditions: control at 15°C, control at elevated temperature (17°C), and nominal concentrations of 5, 50, and 500 µg/L BPA (resulting in deduced effective concentrations of 0.24, 2.4, and 24.1µg/L respectively , according to chemical analysis and the rates of BPA decay, as reported in Ladewig (2004)), at 15°C.

In each stream 3 enclosures were installed, and 26 precopula pairs of gammarids sampled at the Zschonerbach, a stream near Dresden, Germany, were added to each enclosure. Precopula pairs were used for their synchronization in the reproductive stage and allowed easy sex determination.

Two days before the experiment started, *Gammarus* was collected from the field for use in the experiments. These animals were subsampled, and organisms in the subsample were analysed for histological and biochemical parameters. Then, during the experiment, *Gammarus* were collected from the artificial streams and analyzed 34 days (t_{34}), 69 (t_{69}), and 103 days (t_{103}) after the experiment had started (Fig.1).

At each sampling, respectively one enclosure was removed from each artificial stream and the gammarids were allocated to biochemical and histological investigations. The number of replicates differed according to the sampling time and investigated parameter due to the survival of gammarids and percentage of sexes as shown in Tab. 1. In addition, 200 gammarids of different size were transferred to each stream to investigate effects on the population level as described by Ladewig (2004). These animals were not kept in enclosures but could move unhampered in the streams. At the end of the experiment, specimens of these gammarids were also investigated for the histological and biochemical endpoints to compare the effects between those animals which have been kept in enclosures and those from outside the enclosures. However, these results are not further discussed in this work because high mortality in the enclosures prevented a sufficient number of replicates.

The application of bisphenol A started two days after the introduction of the gammarids into the streams (t_0) (Fig.1). The half-life of BPA in water range between 0.8 and 1.7 days at 15°C (Licht et al.; 2004). To help maintain a steady-state exposure of BPA to *Gammarus*, we added BPA weekly as described in detail also by Licht et al. (2004).

Tab.1. Number of *G. fossarum* individuals per sampling for histology and biochemistry

		histology	hsp90	hsp70
t -2		15	15	9
t 34	C 15°C	10	10	10
	C 17°C	10	16	11
	5 µg/l	5	9	9
	50 µg/l	5	9	10
	500 µg/l	9	8	10
t 69	C 15°C	18	9	10
	C 17°C	4	6	6
	5 µg/l	17	8	9
	50 µg/l	17	9	9
	500 µg/l	16	9	2
t 103	C 15°C	4	3	5
	C 17°C	-	-	-
	5 µg/l	-	-	-
	50 µg/l	5	9	3
	500 µg/l	2	5	1

Numbers shown for histology refer to females exclusively, for hsp90 and hsp70 given numbers refer to both, males and females. C 15°C: Control stream 15°C; 17°C: Control stream 17°C; 5, 50, 500µg/L: BPA concentration for the streams at 15°C; t_{-2}: 1st sampling in the field 2 days prior to the introduction into the streams and the BPA application; t_{34}: 2nd sampling 34 days after BPA application; t_{69}: 3rd sampling 69 days after BPA application; t_{103}: 4th sampling 103 days after BPA application.

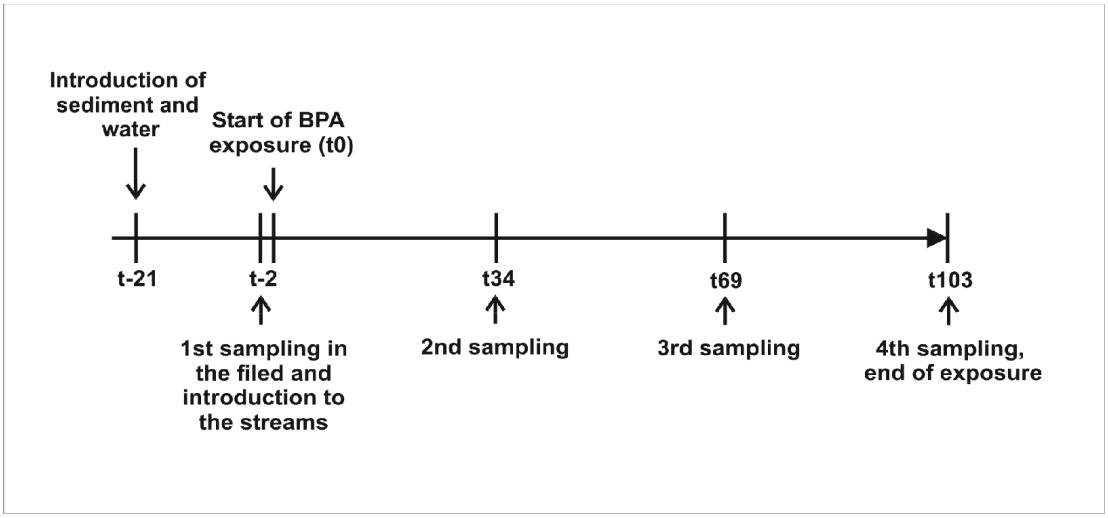

Fig. 1. Time bar for the experimental design, equal for all five artificial indoor streams.

Histological investigations

Gammarids were decapitated and fixed in 2 % glutardialdehyde, dissolved in 0.005 M cacodylate buffer (pH 7.4). The gammarids were decalcified in 5% trichloroacetic acid for two days. The samples where then dehydrated in a graded series of ethanol and finally embedded in Technovit resin (Heraeus Kulzer, Germany). Then, the animals where sectioned as follows: per individual, eight series of each 16 sagittal sections (4µm thickness each) were cut with a Reichert Jung 2050 microtome. The arrangements of the eight section series depended on the individual width of each specimen assuring a complete overview over the histology of each individual. Sections were stained with azur-methylene-blue according to Richardson et al. (1960), and examined using a light microscope (Zeiss, Axioscop 2).

The females were classified as belonging to 6 maturity stages based on the maturity status of their gonads. This was accomplished by examining maturity of the oocytes. The maturity stages of the oocytes were distinguished on the basis of the work of Tan-Fermin and Pudadera (1989) (Fig. 2). Stage-assessment has been verified before in a study relating oocyte development to reproductive stages (Schirling et al., 2004):

- Previtellogenic stage (P): small basophilic cells with a large nucleus, but lacking vesicles.
- Early vitellogenic stage (E): small basophilic cells with few vesicles in the cytoplasm. Occurrence of perinuclear heterochromatin.
- Late vitellogenic stage (L): larger, cubic cells with large vacuoles of lipids or proteins. Follicle cells surround the outer membrane of these oocytes.
- Mature stage (M): This is the last stage of the oocytes before being released to the marsupium. The characteristic egg membrane is surrounding the cell and the ellipsoidal nucleus is located next to the membrane.
- Spent stage (S): Only oocytes which are not released into the marsupium belong to this stage. These oocytes become atretic and undergo lysis.
- In addition, a further maturity "stage" of females was determined which was characterized by oocytes mainly in the spent stage but, in addition, by previtellogenic oocytes of the next reproductive cycle. This was called intermediate stage (SP).

The most developed stage of intact (= non-atretic) oocytes was considered to define the developmental stage of the respective individual. Thus, each individual was classified in one of the stages: SP, P, E, L, M, or S. The developmental stage of all female gammarids at a given time, location, and size class was expressed by a maturity index (mi), according to the following equation using arbitrary coefficients for each oocyte stage: $mi = (x_{SP} + 2x_P + 3x_E +$

$4x_L + 5x_M + 6x_S) *10^{-2}$ where x_{SP}, x_P, x_E, x_L, x_M, and x_S refer to the percentages of the individuals in the respective stages SP, P, E, L, M, and S in the entire sample.

Additionally, both intact and atretic oocytes in the early vitellogenic stage and in the late vitellogenic stage were recorded. To distinguish the types of oocytes from the developmental stage of the entire individual, early vitellogenic oocytes have been abbreviated EVO and late vitellogenic oocytes have been abbreviated LVO. The ratio between atretic and intact oocytes of the EVO and LVO type was calculated for each individual displaying oocytes of at least one of these two types.

Furthermore, the size (= area in the sections) of intact oocytes, of both the EVO as well as the LVO type in the section was determined by morphometric software (Openlab 3.0, Improvision).

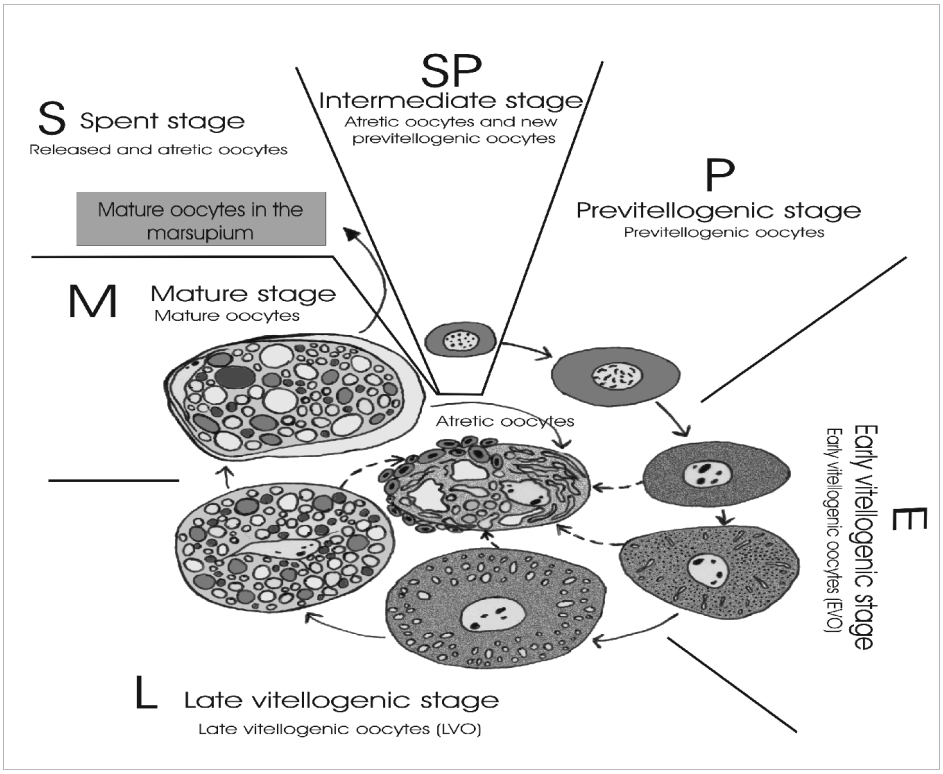

Fig. 2. Different oocyte stages according to Tan-Fermin & Pudadera (1989). Explanation in the text.

Stress protein analysis

For the stress protein (hsp90 and hsp70) analysis, the gammarids were frozen in liquid nitrogen. The animals were individually homogenized in 100 µL extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM Hepes, 2% protease inhibitor Sigma (P8340), pH 7.5), and the homogenate subsequently centrifuged (12 min, 20,000 g at 4°C). Total protein concentration in the supernatant was determined according to the method of Bradford (1976). Constant protein weights (40 µg of total protein per lane) were subjected to minigel SDS-PAGE (12% acrylamid-bisacrylamid) for 20 min at 80 V and 120 min at 120 V in duplicate (samples for hsp90 and hsp70 analysis were run on separate gels). Protein was transferred to nitrocellulose by semi-dry blotting, and the filter was blocked for 2 h in 50% horse serum in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl pH 7.5). After rinsing in TBS, monoclonal antibody (mouse anti-human hsp70; Dianova, Hamburg, Germany, dilution 1:5,000 in 10% horse serum/TBS, or mouse anti-water mold hsp90; StressGen Victoria, Canada, dilution 1:800, respectively) was added, and the filter incubated at room temperature overnight. After repeated rinsing in TBS for 5 min, the nitrocellulose filters were incubated in the second antibody (peroxidase-conjugated goat anti-mouse IgG Dianova, Germany, dilution 1:1,000 in 10% horse serum / TBS) at room temperature for 2 h. After repeated rinsing in TBS for 5 min, the antibody complex was detected by 1 mM 4-chloro(1) naphtol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol. The grey scale values of the Western blot protein bands were quantified using a densitometric image analysis system (Herolab E.A.S.Y., Germany), and related to a hsp90 or, respectively, a hsp70 standard, run in parallel on each gel.

Statistical analysis

For statistical analysis we used the software JMP®4.0 (SAS). Normally distributed data (checked by Shapiro-Wilk's test) were tested for significance with Student's t-test, whereas data with non-normal distribution underwent a Kruskal Wallis test. The α-level for significant differences was set at $p \leq 0.05$. The calculations of the ECx values based on non-linear regression analysis were performed with TableCurve 2D 5.1 (SYSTAT Software Inc.), ECx calculations based on probit analysis were performed with ToxRat Standard 2.09.

RESULTS

Effects of Bisphenol A

In addition to the observations described above, we found effects which can be exclusively related to BPA exposure. As shown in Figure 3a, the maturity indices showed a slight, non-significant trend to higher values, symbolizing an accelerating maturation in BPA-exposed gammarids, regardless of the concentration. As a consequence thereof, the percentages of atretic EVO during the experiment was lower in the BPA treatments than in the control (Fig. 3b). The resulting EC_{10} / EC_{20} values are shown in Tab. 2; as EC_{50} values could have been determined by extrapolation only, these data are not presented. Furthermore, at t_{34}, a concentration-dependent decrease of EVO area was apparent (Fig. 4). High variability, however, prevented significance of these results. Corresponding EC_{10} / EC_{20} values are shown in Tab. 2.

At the same time (t_{34}), the hsp90 levels in gammarids from all three BPA treatments showed a significant decrease in comparison to those from the control stream (Fig. 5a). At $t_{69,}$ this decrease was still visible in the streams treated with 50 µg/L and 500 µg/L BPA (Fig. 5b). EC_{50} values are presented in Tab. 2. The difference in the EC_{50} values on t_{69} are a result of the two calculation methods, non linear regression and probit analysis.

Tab.2. ECx calculations of selected parameters

	non-linear regression			probit analysis		
	EC_{10}	EC_{20}	EC_{50}	EC_{10}	EC_{20}	EC_{50}
atretic EVO t_{34}	0.94	1.63	n.d.	0.48	2.24	n.d.
atretic EVO t_{69}	0.03	0.12	n.d.	n.d.	n.d.	n.d.
area EVO t_{34}	0.44	0.95	n.d.	0.13	0.93	n.d.
hsp90 t_{34}	n.d.	n.d.	0.2	n.d.	n.d.	n.d.
hsp90 t_{69}	n.d.	n.d.	1.72	4.9	8.9	27.9

Calculation of EC_{10}, EC_{20}, and EC_{50} values for three parameters at different sampling times. t_{34}: 2nd sampling 34 days after BPA application; t_{69}: 3rd sampling 69 days after BPA application. Calculation based on effective concentrations with non-linear regression and probit analysis. n.d.: not determined.

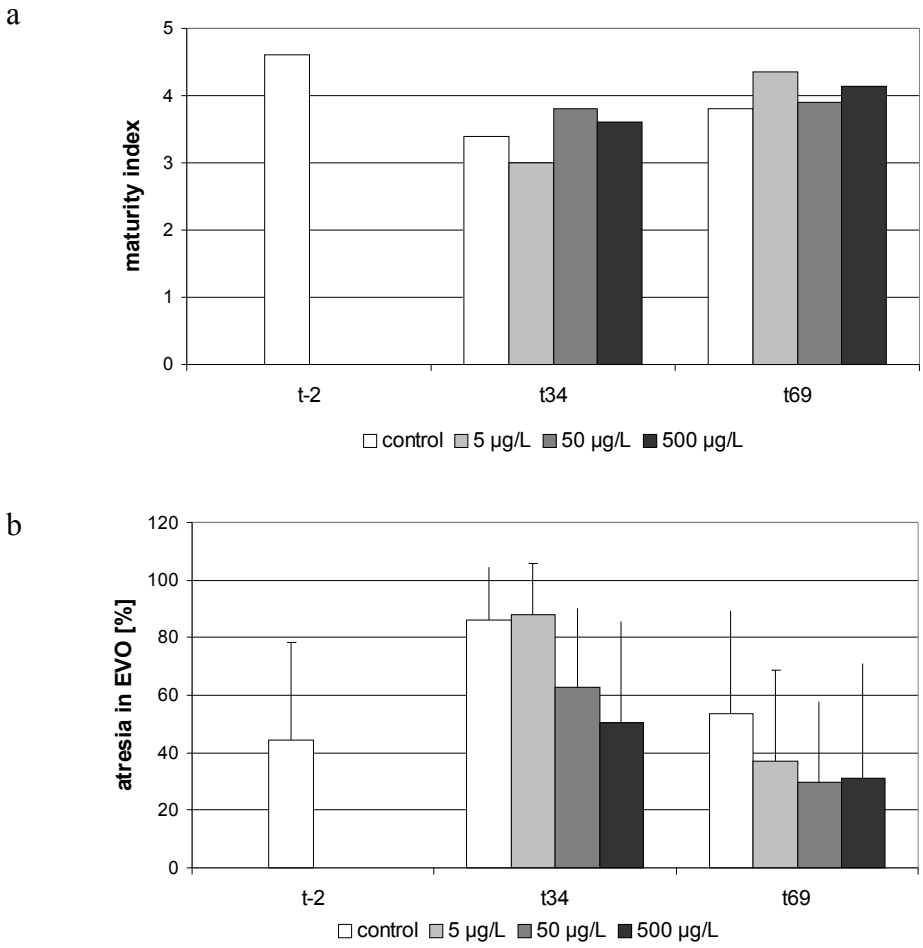

Fig. 3. (a) Maturity indices (means) and (b) percentages of atretic EVO (means ± SD) in the three BPA treatments (5µg/L, 50µg/L, 500µg/L) in comparison to the control at days 34 and 69 of BPA exposure.

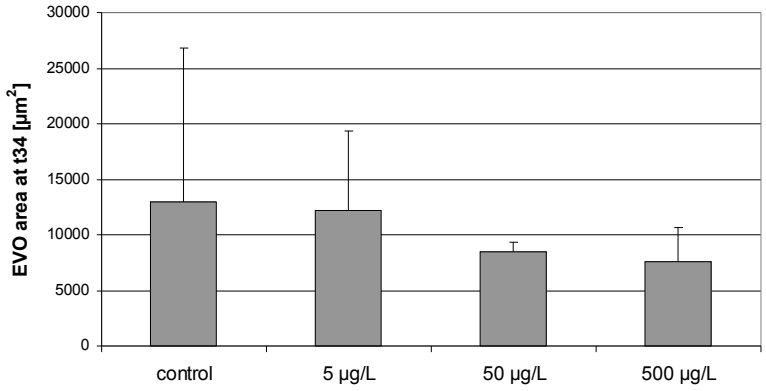

Fig 4. Area of early vitellogenic oocytes (EVO) in sections of *G. fossarum* exposed to one of the three BPA treatments (5µg/L, 50µg/L, 500µg/L) in comparison to the control at day 34 of exposure (means ± SD).

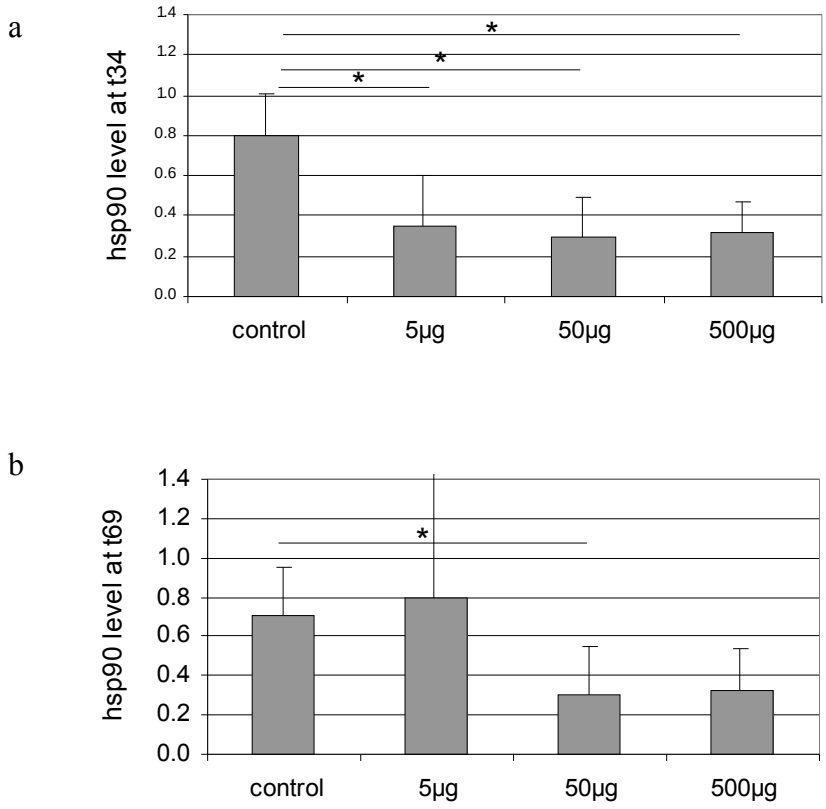

Fig. 5. Levels of hsp90 at day 34 (a) and day 69 (b) of the experiment measured in gammarids exposed to one of the three BPA treatments (5µg/L, 50µg/L, 500µg/L) in comparison to the control stream. Means ± SD. *: significant at $p \leq 0.05$

Captivity effects

During the course of the experiment, mortality of the gammarids in the enclosures increased with proceeding exposure time. Particularly at t_{103}, mortality was 100% in the 17°C control and the stream spiked repeatedly with 5 µg/L BPA (nominal). Also in the control at 15°C, mortality after 103 days was more than 76%. Histological investigations of early vitellogenic oocytes (EVO) revealed a constant decline of the cell area in the control group from t_{-2} to t_{103}, even though the results were not significant (Fig. 6). At the same time, the levels of hsp70 increased, in part significantly, in the control as well as in the BPA-treated streams (Fig. 7).

Due to the high mortality in the enclosures in several streams at t_{103}, biochemical and histological data, obtained from survivors, might be highly artificial and will not be presented later.

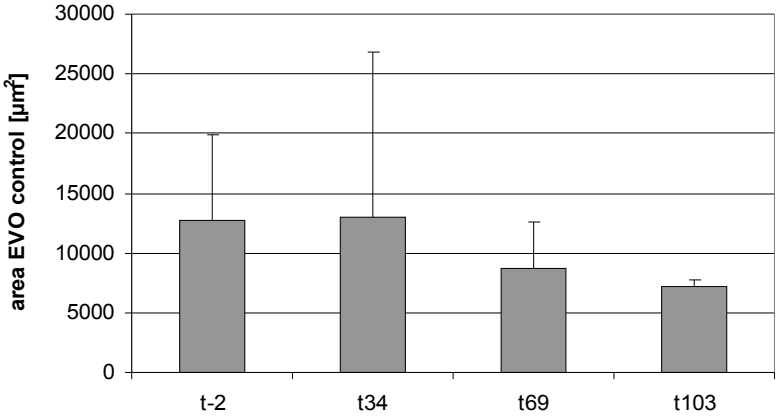

Fig. 6. Area of early vitellogenic oocytes (EVO) in sections of control animals at the respective samplings (means ± SD). t_{-2}: 1st sampling 2 days prior to experiment; t_{34}, t_{69}, t_{103}: sampling at days 34, 69 or 103 of the experiment, respectively.

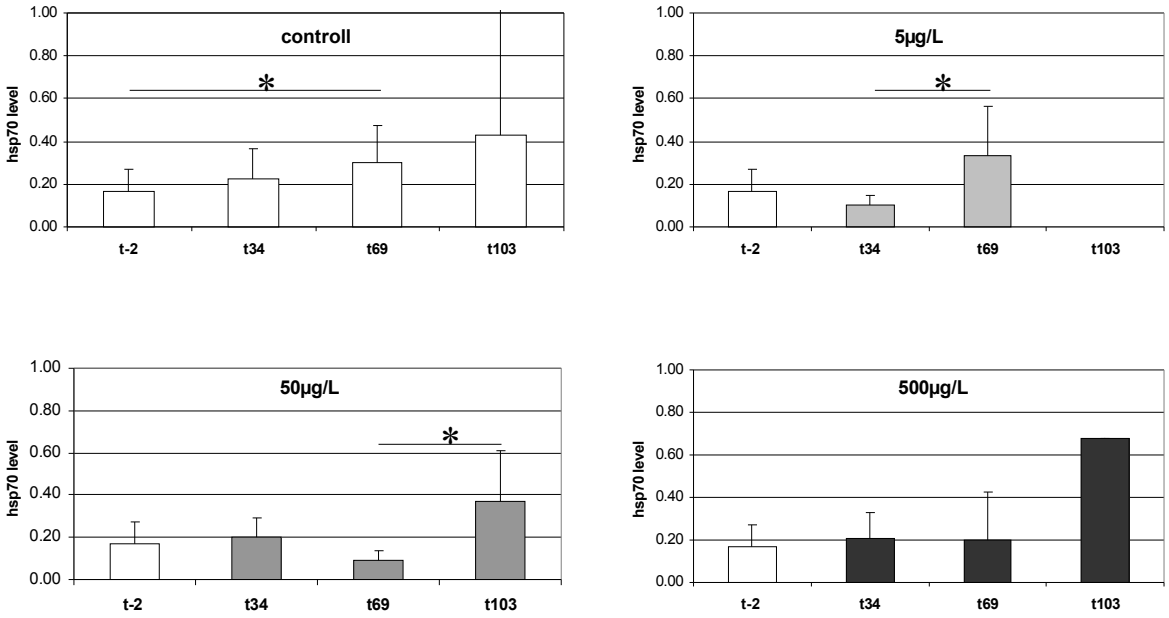

Fig. 7. Hsp70 levels (means ± SD) at the four samplings, illustrated separately for the four streams run at 15°C: 0µg/L (control), 5, 50 and 500µg BPA/L, respectively. t_{-2}: 1st sampling 2 days prior to the experiment; t_{34}, t_{69}, t_{103}: sampling at days 34, 69 or 103 of the experiment, respectively. * significant at $p \leq 0.05$.

Temperature effects

The hsp70 level of animals in the control at 15°C increased only slightly from the start of the experiment on day t_{-2} to the end on day t_{69}. A more pronounced elevation could be observed for gammarids kept under 17°C (Fig. 8a). The maturity index shows a similar pattern. Indices obtained for gammarids kept at 17°C were higher than for those animals kept at 15°C in the samplings at t_{34} and t_{69}, respectively (Fig. 8b).

Table 3 gives an overview of all parameters investigated during the experiment.

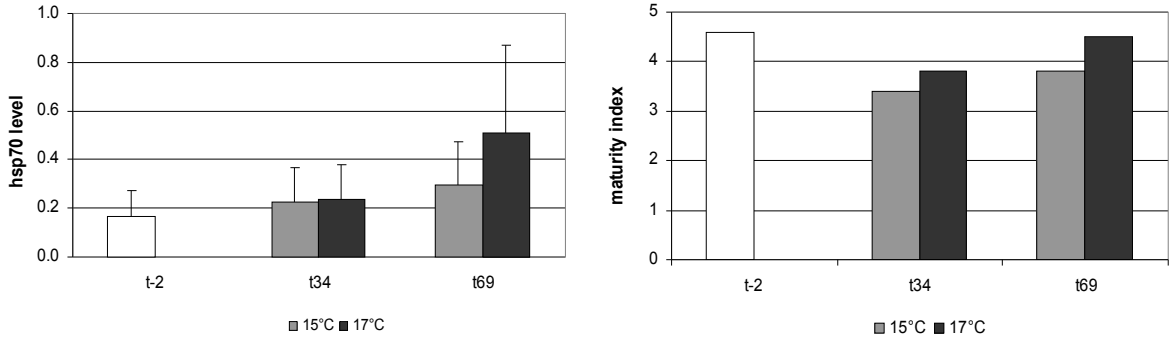

Fig. 8. (a) Hsp70 levels (means ± SD) and (b) maturity indices (means) in gammarids from the untreated streams at 15 °C and 17°C, respectively.t_{-2}, t_{34}, t_{69}: sampling times (see Fig.1).

DISCUSSION

In this study, we showed effects of BPA on gammarids, but also influences of exposure conditions and temperature. We have demonstrated that the used artificial indoor stream system in combination with the enclosures did not result in stress symptoms for at least 69 days which accounts for the reliability of the results obtained within this time span. However, for longer exposure (103d), several parameters indicate adverse changes to the conditions in the enclosures. The level of the stress protein hsp70 increased in animals from all streams during the experiment and shows its maximum value after 103 d, indicating continuous necessity for adaptation to proteotoxicity even in the surviving individuals. The mortality also has its maximum at day 103; at this time only 15% of the originally introduced animals in the control stream were alive (for t_{69}: >70%) in comparison to the initial number of gammarids.

Tab. 2. All results obtained from the experiments performed in the artificial indoor streams

	control 15°C	control 17°C	BPA 5µg/L	BPA 50µg/L	BPA 500µg/L
hsp70					
t_{-2}	0.17 ± 0.10	0.17 ± 0.10	0.17 ± 0.10	0.17 ± 0.10	0.17 ± 0.10
t_{34}	0.23 ± 0.14	0.23 ± 0.14	0.10 ± 0.04	0.20 ± 0.09	0.20 ± 0.13
t_{69}	0.30 ± 0.18	0.51 ± 0.36	0.33 ± 0.23	0.09 ± 0.05	0.20 ± 0.22
t_{103}	0.43 ± 0.70	n.d.	n.d.	0.37 ± 0.24	0.68 ± -
hsp90					
t_{-2}	0.47 ± 0.28	0.47 ± 0.28	0.47 ± 0.28	0.47 ± 0.28	0.47 ± 0.28
t_{34}	0.79 ± 0.22	0.46 ± 0.17	0.35 ± 0.25	0.30 ± 0.19	0.32 ± 0.15
t_{69}	0.71 ± 0.24	0.88 ± 0.51	0.79 ± 0.74	0.31 ± 0.24	0.32 ± 0.21
t_{103}	0.37 ± 0.19	n.d.	n.d.	0.40 ± 0.24	0.37 ± 0.14
maturity					
t_{-2}	4.6	4.6	4.6	4.6	4.6
t_{34}	3.4	3.8	3	3.8	3.6
t_{69}	3.8	4.5	4.4	3.9	4.1
t_{103}	3.3	n.d.	n.d.	3.4	3.5
area EVO					
t_{-2}	12773 ± 7180	12773 ± 7180	12773 ± 7180	12773 ± 7180	12773 ± 7180
t_{34}	13008 ± 13839	8180 ± 1563	12230 ± 7060	8457 ± 857	7537 ± 3132
t_{69}	8642 ± 3947	8505 ± 969	9690 ± 3374	8055 ± 2114	14307 ± 21623
t_{103}	7183 ± 617	n.d.	n.d.	8530 ± 1378	7849 ± 206
area LVO					
t_{-2}	190648 ± 39044	190648 ± 39044	190648 ± 39044	190648 ± 39044	190648 ± 39044
t_{34}	39084 ± 15552	63146 ± 32387	n.d.	24304 ± 20202	34344 ± 11030
t_{69}	69225 ± 34340	132675 ± 57791	62413 ± 52115	86524 ± 3571	76808 ± 45856
t_{103}	n.d.	n.d.	n.d.	n.d.	n.d.
atresia EVO					
t_{-2}	44 ± 34	44 ± 34	44 ± 34	44 ± 34	44 ± 34
t_{34}	86 ± 18	30 ± 35	88 ± 18	63 ± 28	50 ± 35
t_{69}	53 ± 36	39 ± 30	37 ± 32	30 ± 28	31 ± 40
t_{103}	29 ± 39	n.d.	n.d.	49 ± 39	n.d.
atresia LVO					
t_{-2}	48 ± 34	48 ± 34	48 ± 34	48 ± 34	48 ± 34
t_{34}	55 ± 43	54 ± 41	100 ± 0	61 ± 26	69 ± 41
t_{69}	59 ± 31	57 ± 43	41 ± 30	35 ± 40	54 ± 41
t_{103}	100 ± 0	n.d.	n.d.	100 ± 0	100 ± 0
animals outside the enclosures at t_{103}					
hsp70	n.d.	n.d.	0.35 ± 0.12	0.59 ± 0.27	0.03 ± -
hsp90	0.53 ± 0.36	n.d.	0.52 ± 0.25	0.49 ± 0.22	0.48 ± 0.21
maturity	3.1	n.d.	3.0	2.9	3.0
area EVO	14903 ± 4061	n.d.	12823 ± 4182	9189 ± 2228	11383 ± 3833
area LVO	238470 ± -	n.d.	n.d.	n.d.	n.d.
atresia EVO	40 ± 33	n.d.	17 ± 18	28 ± 27	9 ± 15
atresia LVO	50 ± -	n.d.	n.d.	n.d.	n.d.

Means ± standard deviation for each parameter, sampling time, and stream investigated inside and outside the enclosures. n.d.: not detected. Units for hsp70, hsp90: optical volume (area x average grey scale value) of immunoblt bands, relative to standard. Maturity: no unity; area EVO, area LVO: μm^2; atretic EVO, atretic LVO: percent

Since physicochemical parameters of the water remained constant throughout the entire experiment (Licht et al. 2004), the reason for the observed high mortality and changes in hsp70 levels may possibly lie in changes of micronutrients or constraints in movement during exposure. This occurred, although captivity experiments with gammarids in artificial water containing micronutrients according to Borgmann (1996) revealed survival and reproduction for 16 weeks (Ladewig, 2004). Supplementary components were only added to the water at the beginning of the experiment and may have changed in composition during the three months. The reduced area of early vitellogenic oocytes can be seen as the result of a shortage in energy supply due to a stressful environment.
The comparison of the two untreated streams with different temperature regimes (15°C and 17°C) confirmed our expectations. The slight temperature increase of 2°C was mirrored by an increased level of the heat shock protein hsp70 after 34 days and, even more pronounced, after 69 days. Moreover, the maturity index at t_{34} and t_{69} in the 17°C stream was elevated. This is in agreement with observations on brood development time in Pöckl and Humpesch (1990). Depending on the sampling site, the weather conditions and the time of day, the temperatures in streams can differ by a few degrees. Consequently, the effects of slight differences in temperature should be considered in the design and analysis of future field experiments.

Due to the experimental design of this study, the effects of BPA on *G. fossarum* could be demonstrated disregarding the above mentioned parameters ‘captivity’ and ‘temperature’. In this study, the histological investigations revealed two major effects caused by BPA, which were visible in all of the three concentrations after 34 d or, respectively, after 69 days of exposure. Except for the 5 µg/L BPA stream at t_{34} (which showed a single nitrite peak between days 9 and 11 eventually having resulted in a developmental delay and other stress effects in this stream) all samplings showed the maturity indices of the treated animals to be higher than those of the control at the same respective sampling time. This indicates a faster maturation of the oocytes in the female gonad and a shortened reproductive cycle for *G. fossarum* induced by BPA. Similar effects were described by Oehlmann et al. (2000) for two aquatic gastropods, who reported an enhancement of spawning mass and egg production in *Marisa cornuarietis*, and a raised oocyte production in *Nucella lapillus*, after exposure to BPA. Furthermore, Andersen et al. (1999) reported an increased egg production in the copepod *Acartia tonsa* induced by BPA. Duft et al. (2003) found an increased embryo production in BPA-treated specimens of the mudsnail *Potamopyrgus antipodarum*.

Corresponding effects (especially a higher maturity index) were observed in a very recent field study with gammarids sampled at a site downstream of a sewage treatment plant (Köhler and Triebskorn, 2004; Schirling et al., 2005).

Temporally and spatially concomitant to the observed accelerated maturation of female gammarids, we found a decreased number of atretic early vitellogenic oocytes. The presence of atretic oocytes in the ovary of gammarids is a well-known phenomenon: at the beginning of the reproductive cycle. Generally more oocytes are formed than can be released into the marsupium at the end of the cycle. This causes a distinct percentage of oocytes to undergo atresia during their development and growth. An explanation for the reduced number of surplus oocytes, which became atretic during advanced maturation, could result from energetic constraints. Since female gammarids responded to the presence of BPA with a faster reproduction, the elevated energetic expense may have been compensated by a shortage in the numbers of oocytes per reproductive cycle. This response is also demonstrated by a reduced offspring of females after 103d in the streams with 50 and 500µg/L BPA (Ladewig, 2004). Consequently, this would have resulted in a decreased number of atretic cells as well. Furthermore evidence of this theory is provided by the measurements of size of early vitellogenic oocytes. On day 34, particularly the females in the streams treated with 50µg/L and 500µg/L BPA showed a decreased EVO size compared to the controls. This supports our interpretation that accelerated maturation of oocytes resulted in higher costs of energy, which could be at least partially compensated by a reduced size of the developed oocytes.

The biochemical analysis revealed the hsp70 and hsp90 levels to show divergent patterns. Whereas the hsp70 levels of gammarids from the BPA treated streams did not differ from the control in a uniform way, the hsp90 levels in BPA exposed animals showed a clear reduction at t_{34} and t_{69}. The fact that the stress marker hsp70 integrating overall proteotoxicity and, therefore, applied in this study as an 'unspecific stress effect control' for hsp90 did not show any significant changes, led us to conclude that the significant decline of hsp90 was likely due to specific BPA effects. However, since mechanistic studies on the interaction of potential xenohormones with the endocrine system in crustaceans are widely lacking (de Fur et al., 1999), we compare our results with studies of other species. As known for vertebrates, hsp90 modulates the binding of the estrogen receptor to its cognate DNA (Sabbah et al., 1996) and can therefore be seen as a biomarker for endocrine active substances. In mouse uterus for example, it was found that BPA was able to change the level of hsp90 in a dose-dependent way (Papaconstantinou et al., 2001). In this context, it is unclear why the hsp90 level of

gammarids was reduced under the influence of BPA, whereas other studies showed an increase as a result of estrogenic stimulation (Shyamala et al., 1989; Ramachandran et al., 1988). Nevertheless, our finding is in agreement with earlier results from field studies, where gammarids were exposed to water of sewage plant effluents with an elevated endocrine potential (Schirling et al., 2005). In these cases, effluent exposed *G. fossarum* also showed a reduced level of hsp90 in comparison to individuals from the less polluted sites.

In summary, our present study has revealed effects which can be related exclusively to BPA exposure. Even though endocrine disruption could not be mechanistically linked to the impact of BPA, support of causal criteria give some evidence for an interaction of BPA with maturity development in *G. fossarum*. The 'strength of association' criterion was supported by the fact that BPA-induced changes were visible in both, histological and biochemical parameters, whereby the endpoints ‘maturity index’, ‘atresia of EVO’, ‘area of EVO in sections’, and ‘hsp90 level’ were most significant. Furthermore, results obtained from indoor streams with different concentrations of BPA were in line with one another. 'Consistency of association' was achieved by the observation that these results, acquired from artificial indoor stream exposure were in line with those obtained for sewage plant effluents in the field (Schirling et al., 2005), even though these effluents usually contain a mixture of various substances.

'Coherence of association and biological plausibility' is given since BPA was shown to interact with the endocrine system in a number of vertebrates (Stocker et al., 2003; Schönfelder et al., 2002) and since estrogen responsiveness was shown also for the genus *Gammarus* before (Watts et al., 2002). In addition, the 'Specifity of association' criterion was fulfilled by the observation that responses to BPA-containing indoor streams could not be achieved by another stressor, like temperature elevation.

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Kapitel 4

INTERSEXUALITY IN *GAMMARUS FOSSARUM* KOCH, 1835 (AMPHIPODA)

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ABSTRACT

We present the first description of intersexes in *Gammarus fossarum* Koch, 1835 (Amphipoda). Intersexes were found in monthly sampIes collected in two streams in different regions of Germany. The frequency of intersexuality differed considerably between the populations from the two streams. The Lockwitzbach, located in Sachsen, showed a frequency of 8.8% intersexes, which is much higher compared to the Körsch (located in Baden-Württemberg) with 0.6%. In contrast to the Körsch, there were also differences between the two sampling sites in the Lockwitzbach. No seasonal pattern in intersex frequency was observed. Intersexes occurred in all length classes of the adolescent and adult gammarids examined. They were significantly larger than females, but showed no difference in body length compared to males. External sex characteristics of intersex individuals have been described in detail, while in histological analyses of internal sex characteristics ovarian tissue was found exclusively. Microsporidians, parasites often associated with intersexuality, were not detected. The intersexes seemed to be functional females, regarding their ability to breed.

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ZUSAMMENFASSUNG

Wir legen die erste Beschreibung von Intersexen bei *Gammarus fossarum* Koch, 1835 (Amphipoda) vor. Intersexe wurden in monatlichen Proben gefunden, die in zwei Bächen in verschiedenen Regionen Deutschlands gesammelt wurden. Die Häufigkeiten der Intersexualität zwischen den Populationen der beiden Bäche unterschieden sich erheblich voneinander. Der in Sachsen gelegene Lockwitzbach wies eine Häufigkeit von 8,8% Intersexe auf. Dies war im Vergleich zur Körsch (in Baden- Württemberg) mit 0,6% sehr viel höher. Im Gegensatz zur Körsch gab es im Lockwitzbach auch Unterschiede zwischen den Probenahmestellen. Ein saisonales Muster in der Häufigkeit von Intersexen wurde nicht beobachtet. Intersexe waren in allen Längenklassen der untersuchten adoleszenten und adulten Gammariden vorhanden. Sie waren signifikant größer als Weibchen, zeigten jedoch verglichen mit Männchen keinen Unterschied in der Körperlänge. Äußere Geschlechtsmerkmale von Intersex-Individuen werden im Detail beschrieben. In histologischen Untersuchungen von inneren Geschlechtsmerkmalen wurde ausschließlich Ovariengewebe gefunden. Microsporidien, die in Gammariden parasitieren, werden oft mit Intersexualität in Verbindung gebracht. In den untersuchten Geweben konnten sie nicht nachgewiesen werden. Da Intersexe zur Brut fähig waren, handelte es sich bei ihnen um funktionelle Weibchen.

INTRODUCTION

Intersexuality is the occurrence of male and female sex characteristics on the same individual of a normally gonochoristic species. This phenomenon is widely recorded amongst crustaceans (e.g., Munro, 1953; Ginsburger-Vogel, 1975; Legrand et al., 1987; Moore & Stevenson, 1991; Sangalang & Jones, 1997) and has previously been described for some gammarid species (Amphipoda). Buikema et al. (1980) found that about 60% of all females of *Gammarus minus* Say, 1818 possessed penial papillae which are the typical secondary sex characteristics of males. The proportion of intersexes in populations of other gammarid species is generally much lower. Intersex individuals have been reported from populations of *G. duebenii* Lilljeborg, 1851 (cf. Bulnheim, 1965; Dunn et al., 1994), *G. chevreuxi* Sexton, 1913 (cf. Sexton & Huxley, 1921), *G. fasciatus* Say, 1818 (= *G. tigrinus* Sexton, 1939), *G. pulex* L., 1758 (cf. Hynes, 1955), *G. pulex* subterraneus Schneider, 1885 (cf. Anders, 1957),

G. pseudolimnaeus Bousfield, 1958 (cf. Hynes & Harper, 1972), *G. lacustris* G. 0. Sars, 1863 (cf. Ökland, 1969), and *G. pungens* padanus Maccagno & Cuniberti, 1956 (cf. Maccagno & Cuniberti, 1956).

However, intersexuality in *Gammarus fossarum* Koch, 1835 has not yet been reported, though this species is widespread over Europe and highly abundant. *G. fossarum* has been investigated over several decades concerning its taxonomy (Schellenberg, 1942; Wautier & Roux, 1959; Roux, 1971; Goedmakers, 1972), production biology, and ecology (e.g., Lehmann, 1967; Meijering, 1971, 1972; Pöckl & Humpesch, 1990; Pöckl, 1992, 1995). Here we present the first discovery of intersexes in *G. fossarum* populations of two streams in Germany. Next to external sex characteristics, gonads of intersexes were examined histologically.

MATERIAL AND METHODS

The population structure of gammarids was investigated during an effect and exposure monitoring project in two streams in Germany, the Lockwitzbach and the Körsch. The Lockwitzbach is a small, low mountain range stream in Sachsen, whereas the Körsch, a morphologically comparable stream, is situated in Baden-Württemberg. At each stream, one sampling site upstream of a sewage treatment plant effluent and one downstream were established (Lockwitzbach sampling sites: L1 and L2; Körsch sampling sites: Kl and K2). In both streams, sampling sites were about six kilometres apart from each other. Gammarids were sampled approximately every four weeks from July to October 2000 and in April and May 2001 in the Lockwitzbach, and from April to August 2000 in the Körsch. Sampling of animals took place in a river transect using one of the (two) Surber samplers modified by Metag (cf. Metag, 2000). Gammarids were separated from the samples by hand, preserved in 75% ethylalcohol (EtOH) and examined using a Wild dissecting microscope (nowadays by Leica) with a magnification up to 30 x. The species was identified as *Gammarus fossarum* and distinguished from *G. pulex* by the length of the inner ramus of the third uropod, which is only about half as long as the outer ramus, as well as by the setation of the second antenna (Heinze, 1932; Schellenberg, 1942; Wautier & Roux, 1959; Goedmakers, 1972). Sex identification was carried out according to Heinze (1932) and Schellenberg (1942). Female gammarids possess four pairs of broodplates (oostegites) in the thoracic region of the body. Males bear two penial

papillae between the last pair of walking legs. Intersexes were identified by the simultaneous presence of oostegites and penial papillae (Buikema et a1., 1980; Dunn et al., 1994). Furthermore, the type of the hand of the first gnathopod, which is stronger, more elongated, and of a different shape in males than in females, was characterized according to Goedmakers (1972). The occurrence of eggs or juveniles in the brood pouch of intersexes was also recorded. A Wild dissecting microscope (magnification 6x), a CCD-camera (Panasonic), and the image processing program DIGITRACE (Imatec) were used to measure the total body length from the base of the first antenna to the base of the telson. Voucher specimens have been deposited at the Institute of Hydrobiology, Dresden University of Technology, Dresden.

Several specimens of these samples were dehydrated in 96% EtOH for at least 1 hour and then in 100% EtOH for 15 min., critical point dried, mounted on electron microscope stubs, sputter-coated with gold, and examined with a LEO 420 scanning electron microscope.

For histological analyses, 20 garnmarids fixed for 6-8 months in 75% EtOH and determined as intersexes, were transferred after decapitation from EtOH to a 2% glutardialdehyde solution (dissolved in 0.005 M cacodylate buffer, pH 7.4). After three days, they were rinsed in 0.005 M cacodylate buffer (pH 7.4) and decalcified in four portions of 5% trichloroacetic acid (Merck) for two days. The samples were then dehydrated in a graded series of ethanol and embedded in technovit resin (Heraeus Kulzer). Each animal was entirely cut in series of 4 µm sagittal sections on a Reichert Jung 2050 microtome. Sections were stained with Methylene blue-Azur II (Richardson et al., 1960) and were finally examined using a light microscope (Zeiss, Axioscop 2). Data were statistically treated with the Mann-Whitney U-test (comparison of body lengths) or the χ^2 test (significance at $p < 0.05$) using the software package STATISTICA 5.0.

RESULTS

Of 1,002 adult or adolescent *Gammarus fossarum* from the Lockwitzbach, 88 (8.8%) were intersex (table I). With an intersex frequency of 13.3% at site L1 and 7.8% at site L2, respectively, there was a significant difference between the sampling sites of this stream ($P = 0.02$). The frequency of intersexes in adult or adolescent gammarids varied during the sampling period between 3.7 and 17.4%, but showed no seasonal pattern. The frequency of intersexes was neither significantly correlated with the ambient temperature nor with the

varying day length during the sampling period (data not shown). In July 2000, no intersexes were found, but the samples size of adult and adolescent gammarids was very small N = 2 at L1 and N = 9 at L2, respectively). The smallest intersex specimen determined had a body length of 5.5 mm, the largest one was 15.0 mm in length. Within this range, no lack of intersexes in a specific length class occurred. Most of the intersexes were between 7 and 11 mm in length. Median body length of intersexes was 9.1 mm. Compared to the females of the same samples (N = 579; median va1ue = 8.5 mm), intersexes showed a significantly larger total body length P = 0.0003). In contrast, intersexes showed no significant differences in body length compared to males.

Unlike the comparatively high frequency of intersexes in the gammarid populations of the Lockwitzbach, there were only few intersex specimens in the samples from the Körsch. Among 519 animals from the Körsch, only 3 intersexes were found, corresponding to the very low frequency of 0.6%. Hence, no significant difference in intersex frequency between the sampling sites of the Körsch was found.

Like typical females, the intersexes examined possessed four pairs of oostegites, which in mature individuals were often fringed with long setae to form the brood pouch or marsupium. Simultaneously, they had one or two penial papillae, which were of the same shape and size as in typical males (fig. 1). Also, some of the males possessed only one penial papilla: a total number of 18 intersexes and 3 males collected from the Lockwitzbach had only one penial papilla. There was a significantly higher percentage of intersexes with only one penial papilla

Tab. 1. Intersex abundance and frequency in adult or adolescent *Gammarus fossarum* Koch

Sampling site	Total number of speciments	Number of intersexs	% intersexes
Lockwitzbach	1,002	88	8.8
L1	173	23	13.3*
L2	829	65	7.8*
Körsch	519	3	0.6
K1	114		0.9
K2	405	2	0.5

Ll/L2, sampling sites of the Lockwitzbach, upstream/downstream of a sewage treatment plant
Kl/K2, sampling sites of the Körsch, upstream/downstream of a sewage treatment plant.
*, significant difference, with $p < 0.05$.

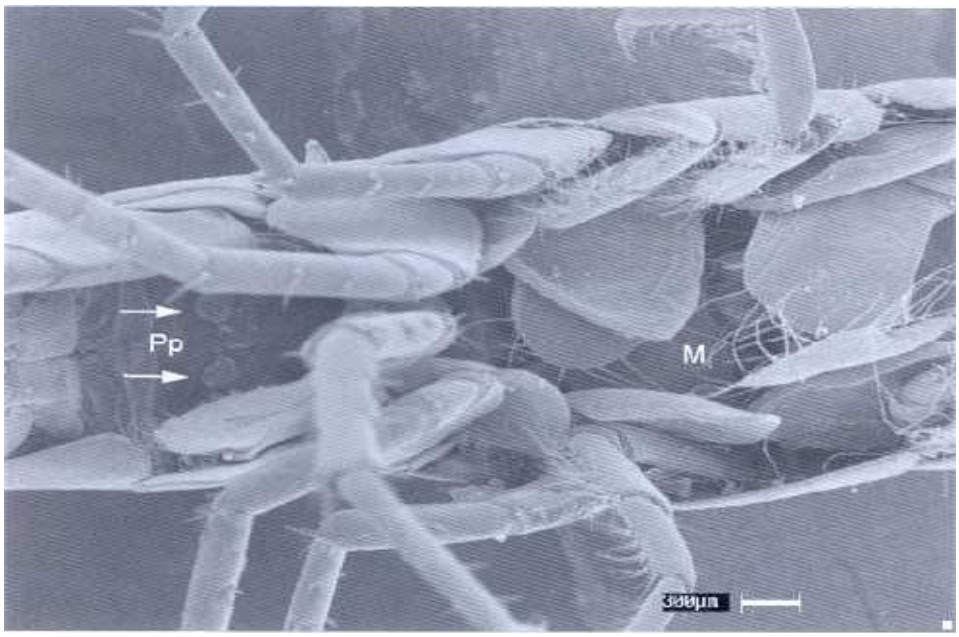

Fig. 1. Scanning electron micrograph of an intersex of *Gammarus fossarum* Koch, 1835, showing the marsupium formed by oostegites. The penial papillae can be seen between the last pair of peraeopods. M, marsupium; Pp, penial papillae.

compared to males with only one penial papilla (20.5% and 0.9%, respectively; $p < 0.0001$). As reported above for the frequency of intersexes in total, there was a tendency to a higher frequency of intersexes with one penial papilla at L1 (7 individuals) compared to L2 (11 individuals) (30.4% and 16.9%, respectively), even though this was not significant. While all three males with one penial papilla were retrieved from site L1, the frequency of such individuals was significantly higher at L1 in comparison to L2 (4.4% and 0%, respectively; $p = 0.006$). All males examined from the populations of the Körsch possessed two penial papillae, but one of the three intersex specimens had only one such papilla.

Apart from the secondary sex characteristics normally present in one sex only, there are other structures showing sexual dimorphism as well. Therefore, the type of the first gnathopods of 71 intersexes was determined. Most of these were female-like, only three individuals from sampling site L2 exhibited roughly male-like first gnathopods.

One intersex individual from the Lockwitzbach was found in precopula, being carried by a male, thus showing the mating behaviour of a functional female. Sixteen intersexes of the Lockwitzbach and two intersexes from Körsch samples were ovigerous. Two specimens found in the Lockwitzbach carried juveniles in their brood pouch.

In 17 of the 20 intersexes that were histologically examined, exclusively ovarian tissue could be identified (fig. 2). In three animals no gonadal tissue was found at all, most probably due to an inadequate fixation by ethanol. The female gonads showed different developmental

stages of oocytes, including previtellogenic stages and mature stages. The amount of atretic oocytes, however, did not differ between normal females and intersexes (Schirling, pers. comm.). In none of the specimens male gonadal tissue or spermatocytes were discovered. No microsporidians, often associated with intersexuality, could be found, either.

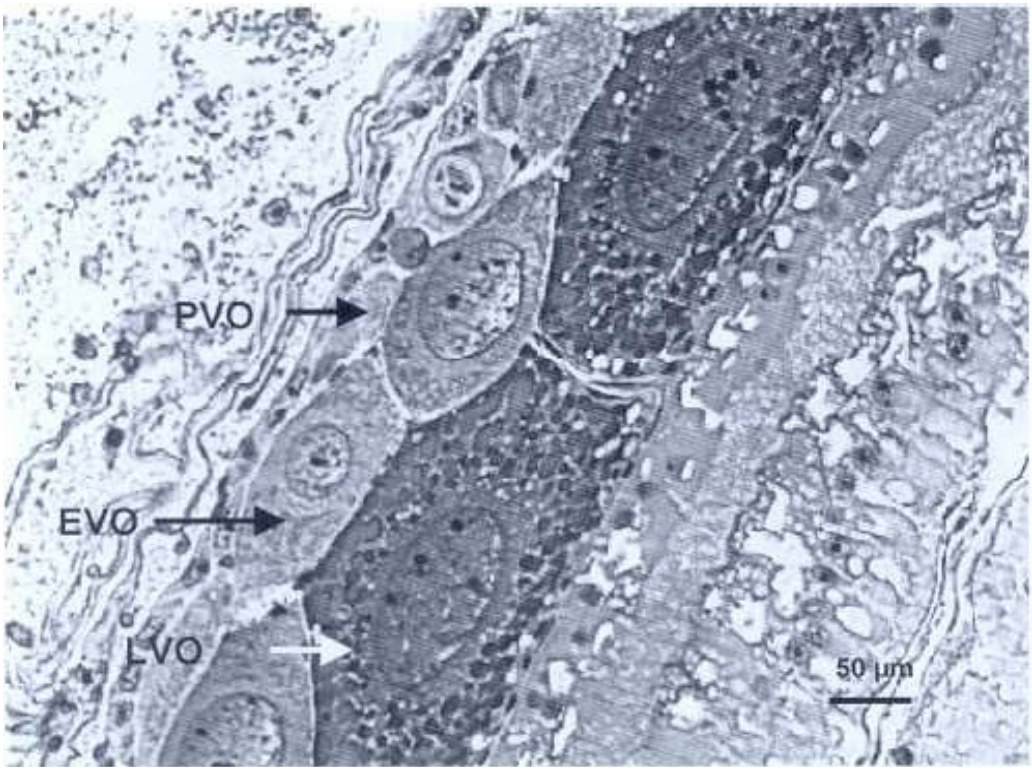

Fig. 2. Sagittal section through gonadal tissue of an intersex of *Gammarus fossarum* Koch, 1835, stained with Methylene blue-Azur II. EVO, early vitellogenic oocyte; LVO, late vitellogenic oocyte; PVO, previtellogenic oocyte.

DISCUSSION

In this study, a remarkably high frequency of intersexes was encountered in the populations of *Gammarus fossarum* in the Lockwitzbach, with overall 8.8%. This value is of approximately the same level as that found in a British *G. duebenii* population (10.8%; cf. Dunn et al., 1994). However, in the Körsch, intersex frequency was much lower (0.6%), resembling rather the observations of Bulnheim (1965) in a German *G. duebenii* population where intersexes were rare as well (0.5%). Sexton & Ruxley (1921) recorded in laboratory strains of *G. chevreuxi* intersexes of a larger size in comparison with normal adults of almost the same age.

Therefore, the body length of gammarids frorn the Lockwitzbach is of special interest. Although age could not be taken into account, intersexes had a similar median body length compared to males, but were larger than females. Dunn et al. (1994) found no significant differences in body weight of intersexes compared to females in *G. duebenii*. This is in contrast to our results. Although Dunn et al. (1994) investigated the body weight, the results could be compared with body length because of the signiticant correlation between these two variables shown by Pöckl (1990).

In our investigations, intersexes of *G. fossarum* possessed oostegites and penial papillae of normal size and morphology. This is in accordance with the results of Dunn et al. (1994) on *G. duebenii*. In *G. pseudolimnaeus* (cf. Rynes & Rarper, 1972), *G. pulex*, and *G. fasciatus* (cf. Rynes, 1955), intersexes had reduced oostegites, and in *G. minus* penial papillae of intersexes were only about half the size as those in males (Buikema et al., 1980).

In both streams, intersexes of *G. fossarum* developed one or two penial papillae, a phenomenon that is also known from intersexes of other gammarid species (Bulnheim, 1965; Buikema et al., 1980). On the other hand, the presence of only one penial papilla in gammarid males has not yet been described, but was here found in a few *G. fossarum* males from the Lockwitzbach.

Intersexes of *G. fossarum* seemed to be functional females, because one specimen was found in the position of the female in a precopula pair. Moreover, some individuals carried eggs or even juveniles within the brood pouch. This is in accordance with the findings of Dunn et al. (1994) in a British population of *G. duebenii* and of Buikema et al. (1980) in *G. minus*. However, Bulnheim (1965) classified intersexes of a German population of *G. duebenii* into five different types. Only the strong female type was able to reproduce and the other types were infertile. The femaleness of intersexes of *G. fossarum* was also reflected by their internal histology, because ovarian tissue was found exclusively. This corresponds with the histological results of Buikema et al. (1980) in *G. minus* and those of Bulnheim (1965) in the strong female type intersexes of *G. duebenii*. Some of the intersex individuals in *G. lacustris* had fully developed but setaeless oostegites (Ökland, 1969). Hence, they should not have been able to form a normal marsupium. Intersexes of *G. fasciatus*, of *G. pulex* (cf. Hynes, 1955), and of *G. pseudolimnaeus* (cf. Hynes & Harper, 1972) were considered males by those authors. Intersexes of *G. pungens* padanus possessed male secondary sex characteristics and abnormal gonads that contained spermatocytes and oocytes, but had only a male function (Maccagno & Cuniberti, 1956). A comparison of the various studies shows no

tendency towards one functional sex in gammarid intersexes.

Experimental evidence indicates that sex determination in gammarids is controlled by a polyfactorial system of sex genes as well as by several non-genetic factors (Bulnheim, 1972; Sutcliffe, 1992). Whether a disturbed interaction of male and female genes in an individual may lead to intersexuality is still not clear, however.

Bulnheim (1975) described a feminizing influence of the transovarially transferred microsporidians *Octosporea effeminans* Bulnheim & Vavra, 1968 and *Thelohania herediteria* Bulnheim, 1969 on the host's offspring in *G. duebenii*. He suggested that microsporidian exudates might inhibit the differentiation of the androgenic gland or interfere with the androgenic gland honnone. However, Dunn et al. (1994) stated that parasitism could not be the main cause for intersexuality in this gammarid. Microsporidian infection was not observed in *G. fossarum* intersexes. It should be noted, though, that the animals were fixed in ethylalcohol for sex determination. Thus, they may have been inadequately preserved for the detection of microsporidians. The action of these microsporidians as well as the epigenetic feminizing factor in the amphipod *Orchestia gammarellus* (Pallas, 1766) (Talitridae), is temperature-sensitive (Ginsburger-Vogel, 1975; Bulnheim, 1978), but alterations in intersex frequency due to increasing temperature in the Lockwitzbach or the Körsch were not noted.

Photoperiod has been shown to be another cue for sex determination in *G. duebenii* and in *G. zaddachi* Sexton, 1912 (cf. Bulnheim, 1972). It is significantly related to intersex frequency in the former species when interacting with temperature (Dunn et al., 1996). In our investigations, the different photoperiod regimes during the sampling period were not correlated with the occurrence of intersexes in the Lockwitzbach.

Other environmental factors may be responsible for the induction of intersexuality in gammarids as well, but these still remain unknown. The frequencies and characteristics of intersexes in various garnmarid populations examined in field studies show major differences. It has not yet been clarified, whether these differences can be attributed to the different species or populations, respectively, or whether they depend on the sampling site, for instance, the inherent regime of physical and/or chemical variables. Because sex differentiation is controlled by hormones, endocrine disruption might also lead to intersexuality as shown, for example, for tributyltin in the prosobranchian snail *Littorina littorea* (Linnaeus, 1758) by Bauer et al. (1995). In the Lockwitzbach, the effiuent of the sewage treatment plant probably was not responsible for the induction of intersexuality, as intersex frequency was somewhat higher at the upstream sampling site (L1) than downstream the plant (L2).

Further research is necessary to determine the causative factor(s) for this phenomenon, and to elucidate the mechanism behind the development and the maintenance of duplicate sex characteristics in intersex individuals. Dunn et al. (1990) reported about a lower pairing success of intersexes compared to females, which would reduce fitness. Thus, from an ecological point of view it would be crucial to study the impact of intersexuality on the population dynamics of gammarids.

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Kapitel 5

ESTROGEN RECEPTOR WAS NOT LOST AT THE BASIS OF ECDYSOZOAN EVOLUTION: EXPRESSION IN CRUSTACEA IS STIMULATED BY 17α-ETHINYLESTRADIOL

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ABSTRACT

Within the protostome clade of invertebrate animals, the presence and relevance of a sex steroid-responsive system, including estrogen receptors (ER) remains unclear for the different phyla. Generally, it is believed that ER orthologs have been lost within the Ecdysozoa. Here, we present the identification, sex-specific expression, and induction of an ER ortholog in an ecdysozoan species, the crustacean *Gammarus fossarum*. Female expression of the *ER* gene was proven by targeting a 96 bp motif with high homology to vertebrate *ER* sequences. In addition, two different motifs of the ER amino acid sequence were targeted exclusively in adult females while they were absent in males and juveniles. Exposure to 17α-ethinylestradiol induced the ER level in adolescent females, but failed to stimulate expression in males and adult females. Our data provide evidence that ER protein deriving from a putative ancestral steroid receptor was not lost in a basal group of ecdysozoans.

***Key words*:** Amphipoda, Crustacea, ER alpha, *Gammarus fossarum*, invertebrate endocrinology, sex steroids

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To be submitted

It was suggested, for a long time, that sex steroid receptors (SR) are restricted to the small corner of the animal kingdom represented by vertebrates and the closely related taxa Acrania and Echinodermata. Until the year 2002, no sex steroid receptors were found in any species outside the deuterostome clade, which gave rise to the suggestion that these receptors arose in ancient deuterostome (Thornton 2001). Other nuclear receptors, e.g., ecdysone receptors which are present in arthropods, belong to nuclear receptor clades that seem to have branched off from the ancestral receptor before the origin of the deuterostome sex steroid receptors (Baker 1997). The origin and relevance of estrogens in protostomes still remained unclear even when it was demonstrated that estrogens and other vertebrate type steroids appear to be involved in the reproductive endocrinology of molluscs (di Cosmo, et al. 2002; Oberdorster & McClellan-Green 2002). Just recently, the isolation of an estrogen receptor ortholog from the mollusc *Aplysia californica* and the experimental characterization of functional domains of the ancestral protein from which all extant SRs supposedly have evolved, was reported (Thornton, Need & Crews 2003). However, none of a diverse panel of vertebrate steroid hormones - estrogens, androgens, progestins, and corticoids - activated or repressed the constitutive level of the *Aplysia* ER. Therefore, the authors suggested that, within the protostomes, ligand regulation was lost in the lineage leading to the *Aplysia* ER, and that the gene was lost entirely from the genome during ecdysozoan evolution. Nevertheless, the latter conclusion ignored observations from previous studies suggesting the presence of an estrogen-responding system in ecdysozoans, comprising arthropods and nematodes. In a long-term exposure experiment, populations of the freshwater amphipod, *Gammarus pulex*, showed a shift in the male:female ratio from initially 1:1 to 0.36:1 when exposed to 0.7 µg 17α-ethinylestradiol (EE2) per liter water for 100 d (Watts, Pascoe & Carroll 2002). In two nematodes, *Panagrellus redivivus* and *Caenorhabditis elegans*, estrogen-binding capacity was found (Hood, Calabrese & Zuckerman 2000). However, theses findings alone are not sufficient to conclude on the existence of sex steroid receptors in these species since common eukaryotic cytoplasmic proteins orthologous to the yeast *Old Yellow Enzyme* bind estrogens with high affinity (Madani et al. 1994).

We aimed at identifying an estrogen receptor in the amphipod crustacean *Gammarus fossarum*, an ecdysozoan. A crustacean species was chosen for the ancient origin of this taxon. In order to target potential *ER* mRNA, we designed degenerated primers to amplify a cDNA sequence corresponding to the phylogenetically highly conserved nucleotide sequence which encodes amino acids 361 - 393 of the human ERα. Using these primers, PCR amplification of

cDNA reversely transcribed from mRNA which was isolated from the homogenate of female *G. fossarum* resulted in a 96 bp product with high sequence homology to amniote *ER* genes (81%-90% base pair identity to mammalian (*Mus*, *Rattus*, *Bos*, *Equus*, *Homo*) *ERα*, 85% to *Alligator ERα*) decreasing homology to fish and mollusc *ER* genes (*Danio ERα*: 65 %, *Aplysia ER*: 58%) but only low homology to other arthropod nuclear receptor genes (*Drosophila ERR (estrogen-related receptor)*: 58%, *Drosophila EcR (ecdysone receptor)*: 49%).

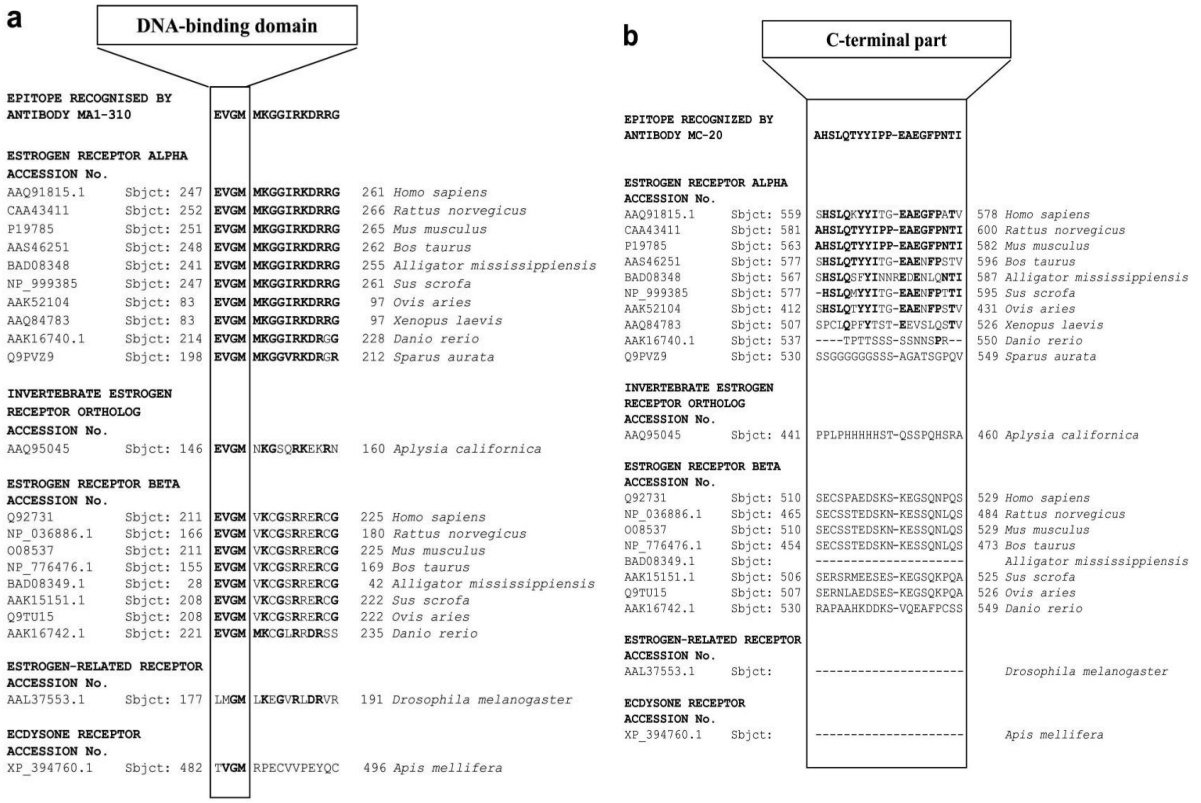

Fig. 1: Alignment of estrogen and ecdysone receptor sequences including the estrogen-related receptor from *Drosophila melanogaster* and the *Aplysia* ER obtained from the *National Center for Biotechnology Information* (NCBI) *Entrez site* in FASTA format, using ClustalX 1.83. **a**: Protein alignment revealed high conservation of ER sequences within and close to the region of the DNA binding domain while similarity of the estrogen-related and ecdysone receptor sequences was lower. Based on amino acid sequence identities (bold letters), a commercial antibody (MA1-310, *Affinity Bioreagents*, Germany) directed against parts of that DBD region (shaded box) revealed highest specificity to ERα. **b**: The C-terminal part of all available ER protein sequences is highly divergent and the antibody MC-20 targets sequences with high structural similarity to the mammalian ERα only.

At the protein level, we created a multiple protein sequence alignment over a range of parameters and considered all available estrogen and ecdysone receptor sequences obtained from the *National Center for Biotechnology Information* (NCBI), including the estrogen-related receptor from *Drosophila melanogaster* and the *Aplysia* ER (Fig. 1a). We identified a sequence within and close to the DBD that shares highest amino acid identities between the different estrogen receptors from different species, screened most of the commercial antibodies for targeting that particular motif, and identified an antibody (MA1-310) which was raised against parts of the DBD. Using MA1-310, immunoblot analyses revealed a specific immunoreactivity against ERα with a molecular weight of approximately 62 kDa only in female *G. fossarum,* but not in male (Fig. 2; n=6, respectively).

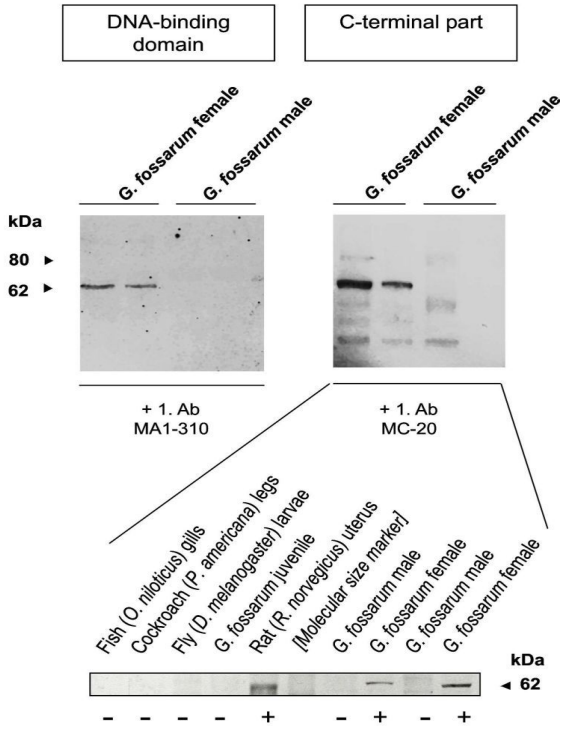

Fig. 2: Identification of an ERα ortholog in female *G. fossarum.* Western blot analysis using the antibodies MA1-310 (directed against a DBD epitope) and MC-20 (directed against an epitope at the C-terminus) both revealed a specific immunoreactivity against ERα with a molecular weight of approximately 62 kDa only in female *G. fossarum*, but not in males. Since MC-20 was raised against the phylogenetically highly variable C-terminal part of the mammalian ERα, further immunoreactivity was only present in the rat (*Rattus norvegicus*) uterus (positive control). No immuno-positive bands were found in larval and juvenile arthropod tissues containing the ecdysone receptor or the estrogen receptor related protein (*G. fossarum* juveniles, *Drosophila melanogaster* larvae, legs of juvenile cockroach *Periplaneta americana*). Fish tissue not expressing ER (tilapia *Oreochromis niloticus* gills) was used as negative control.

In addition, protein alignment revealed the N- and C-terminal parts of all available ER protein sequences to be highly divergent (Fig. 1b), which implies only little evolutionary pressure on their conservation. To further characterize the ER protein of *G. fossarum*, we, therefore, used another specific primary antibody raised against the C-terminal part of the mammalian ERα (MC-20; Fig. 1b). Again, we could demonstrate a specific immunoreactivity against ERα protein with a molecular weight of approximately 62 kDa only in female *G. fossarum*, but not in males and juveniles (Fig. 2; n=10 respectively, and n=9 in juveniles). As expected from the highly divergent C-terminal parts of the different nuclear receptors, we could not detect any other specific immunoreactivity due to cross-reactivity with members of the nuclear receptor clade other than mammalian ERα. No immunoreactivity was found in homogenates of tissues from different species containing the ecdysone receptor, an estrogen-related receptor, the fish ERα, or non-vertebrate ER orthologs (Fig. 2).

Based on the reported demographic effects of EE2 on gammarids (Watts, Pascoe & Carroll 2002), male, female and juvenile *G. fossarum* were exposed to 10 µg EE2 /L for 5 days. EE2 induced ERα protein in adolescent females to levels as high as present in untreated adult females (Fig. 3). In males (no immunoreactivity detected; data not shown) and adult females, EE2 failed to increase ERα protein expression.

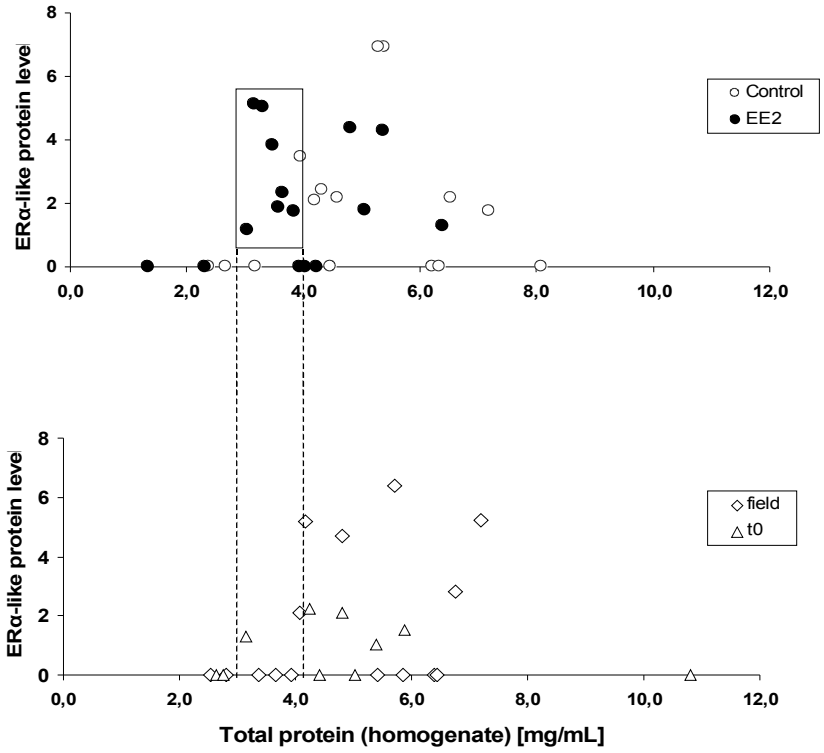

Fig. 3: Induction of the 62 kD ERα protein in females of *G. fossarum* by EE2. ERα protein level vs. individual size (represented by total protein concentration in the homogenate) in 10 µg EE2/L-exposed and solvent (ethanol) control animals (top), and field-caught animals and t0 controls taken priot to exposure (bottom). Individual data are presented. In none of the males, ERα was detected, irrespective of EE2 treatment and the size of the animals which can be used as an estimate on maturity in this species. In females, the ratio of individuals displaying an ERα protein level above the detection limit varied among the four exposure groups: field: 40%; t0 group: 50%, control group: 53%, EE2-exposure: 69%. It is known that female *G. fossarum* turn from adolescence to adulthood at a total body length of about 6.5 mm, even though an exact minimum size for adulthood cannot be given. Since the body length of gammarids which need to be kept continuously frozen can hardly be determined exactly, we used the amount of extractable protein (i.e. the concentration of total protein in the homogenate) as a substitute for the size. In EE2-exposed females, the minimum size required to detect the ERα was 0.23 mg extractable protein, i.e. about 23% lower than in control animals. In that size class, EE2-exposed adolescent females showed high ERα protein levels (rectangle) which were similar to those obtained for far larger, adult females of the other three experimental groups.

Our data on the identification, sex-specific expression, and induction of an ERα orthologous protein in an ecdysozoan species reveal that sex steroid receptors have evolved also in (at least part of the) arthropods and possibly also in other ecdysozoan taxa. In contrast to the ER ortholog from *Aplysia californica* (Thornton, Need & Crews 2003), the gammarid ERα protein seems to be structurally similar to the mammalian ERα and was found to be inducible by estrogen.

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Kapitel 6

AN INVERTEBRATE EMBRYO TEST WITH THE APPLE SNAIL *MARISA CORNUARIETIS* TO ASSESS EFFECTS OF POTENTIAL DEVELOPMENTAL AND ENDOCRINE DISRUPTORS

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ABSTRACT

A novel invertebrate embryo test with the apple snail, *Marisa cornuarietis*, comprising a test protocol for the following developmental endpoints is described: formation of eyes and tentacles, heart rate, hatching, weight after hatching. To evaluate effects on embryonic development, the snails were treated in a first step with 250 µg/L or 500 µg/L cadmium. Sublethal effects in terms of a significant delay in hatching could be found in the 250 µg/L treated animals 500 µg/L Cd were lethal for the snail embryos. To test endocrine disrupting chemicals with this protocol, experiments with bisphenol A (50 µg/L, 100 µg/L) and 17α-ethinylestradiol (10 µg/L) were performed. In both treatments an increase of weight after hatching was observed as well as a significant decline in the heart rate of the embryos. As shown here, the sensitivity of the *M. cornuarietis* embryo test is equal of even higher than other test species like zebrafish embroys, and can therefore be regarded as an alternative or supplement for ecotoxicological studies.

Key words: *Marisa cornuarietis*, embryo test, endocrine disruption, bisphenol A

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INTRODUCTION

Embryo tests or early life stage tests have become important tools for environmental risk assessment in recent years. These tests are able to provide data of high biological relevance at the interface between the individual and the population levels. Furthermore, they can bridge between cell culture experiments whose results are difficult to relate to field situations, and animal studies which provide highly relevant *in situ*-data but with great expense and critical ethical aspects. Advantages of early life stage tests, for example with the zebrafish *Danio rerio*, are their short duration in comparison to life cycle tests and, furthermore, they cover highly sensitive stages of the development. Up to now, there are only few standardized test protocols like *Dar*T or AMPHITOX (Nagel, 2002; Fridman et al., 2004); while tests like these are lacking entirely for invertebrates.

With its largely transparent eggs, *Marisa cornuarietis* provides the opportunity to follow the development of the embryo from outside up to the time of hatching. This allows to find sublethal parameters which are affected by treatment with different chemical compounds. To define these parameters we conducted a first test with cadmium. This heavy metal is a pollutant with well-known effects on the embryonic development of several species (Cœurdassier, 2003; Gomot, 1998; Itow et al., 1998; Eaton et al., 1978) and, therefore, was chosen to select appropriate endpoints in our test design. Further investigations were performed with 17α-ethinylestradiol (EE2) and bisphenol A (BPA) to check for effects which may be attributed to endocrine disruption. For the artificial hormone EE2 used in contraceptiva, many reports on its endocrine activities are present (e.g. Pawlowski et al., 2004; Schultz et al., 2003). Also for BPA diverse effects on the endocrine system have been reported previously (e.g. European Chemical Bureau, 2003; Stocker, 2003; Jobling et al., 2002; Schönfelder et al., 2002; Staples et al., 1998). BPA is produced in large quantities of approximately 2,5 million tonnes per year (Staples et al., 2002), it is a key building block of polycarbonates, and can be found in many products like epoxy resin, dental sealants and composites, as well as in coatings of metal food and beverage cans. So far, only a few studies have reported effects of BPA on invertebrates (Duft et al., 2003; Segner et al., 2003; Pascoe et al., 2002; Jobling et al., 2002; Watts et al., 2001) and the mode of action in these organisms remains still unknown.

The purpose of this study was two-fold: First, It aimed at establishing an invertebrate counterpart to the zebrafish embryotest, in order to use a prosobranch snail which was shown

before to be sensitive to endocrine disruptors as a model organism. On the other hand, this study aimed at assessing the sensitivity of this invertebrate species to a well-known toxin and two endocrine disruptors.

METHODS

Test animals and cultivation

Marisa cornuarietis (Mesogastropoda: Ampullariidae) is a common inhabitant of rivers and estuarines in south and central America. In contrast to other Ampullariidae, it possesses a lung in addition to a monopectinate gill and, furthermore, it deposits its egg masses below the water line, unlike the other species of this family. The egg clutches consist of 20 to 80 eggs which are embedded in a gelatinous mass and stuck to aquaria walls or other objects. Depending on the temperature, the development of the embryo until hatch takes between 8 and 20 days (about 12d at 26°C) and was described in detail by Demian and Yousif (1973).

The parental animals for this experiment were obtained from a breeding stock of the Zoological Institute in Frankfurt a.M., Germany. About 150 adult *M. cornuarietis* were kept in 120 L glass aquaria filled with tap water to which sea salt (hobby-marin, Dohse Aquaristik) was added up to a water conductivity of about 820µS/cm^2. The light regime was adjusted to 12h/12h at a water temperature of 24°C ± 1°C. The parental animals were fed regularly with fish flake food (Hagen, Germany), fresh carrots, and cucumbers.

Exposure conditions

In a first experiment, the developing embryos were treated with two concentrations of cadmium. The aim was to examine the effects of a substance with known embryo toxicity on the development of *M. cornuarietis*, in order to define sensitive parameters for a standardized test design. In a second step the xenohormone bisphenol A was tested for possible effects on the embryo during development. This was accomplished by 17α-ethinylestradiol which is commonly used as a positive control for vertebrate endocrine disruption.

Egg masses were removed carefully from the walls of the aquaria every day, divided with razor blades, and 15 to 20 eggs were distributed to each Petri dish for either control or exposure groups, and incubated at 26°C ± 0,5°C for further investigations. The controls as well as the solutions used for the different treatments were prepared on the basis of the water

of the aquaria. The Petri dishes were covered during the exposure and the control water as well as the solutions were renewed daily.

Cadmium was applied as $CdCl_2$ (Merck, Germany) in a stock solution of 1 gCd/L in aqua bidest. For the test solutions the stock was diluted with water of the aquaria to the final concentrations of 500 and 250 µgCd/L. All tests with cadmium were performed in polyethylene Petri dishes.

For the stock solution of BPA, 100 mg BPA (Fluka, Germany) was dissolved in Aqua bidest (100 mg/L) and afterwards diluted with aquaria water to concentrations of 50 and 100 µgBPA/L. To counter the fast degradation of bisphenol A all solutions including the stock solution were renewed at least every third day.

Ethinylestradiol (EE2, Fluka, Germany) was dissolved in 50% dimetylsulfoxide (DMSO) in aqua bidest to a stock solution of 1 gEE2/L. Both, solvent control (0,005‰ DMSO) and the EE2 treatment (10µg/L) were diluted with aquaria water. For the tests with BPA and EE2 glass Petri dishes were used.

Embryo toxicity test

Once a day, the development of the embryos within the eggs was observed from the day of egg laying until hatching of the young snail. A stereo microscope was used to assess the following endpoints: mortality [%], formation of the eyes [%], formation of the tentacles [%], heart rate[min^{-1}], and hatching [%]. Furthermore, the weight after hatching [mg wet wt.] was recorded. This procedure ensured that every day the record of all parameters have been accomplished for each Petri dish. For each treatment 9 replicate Petri dishes were investigated (n=9).

The weight of the animals was measured after transfer of the newly hatched snails from the Petri dishes to the surface of soft paper tissue. After one minute the adhesive water was removed completely and respectively five individuals were pooled and weighed at a time on an analytical balance.

Statistical analysis

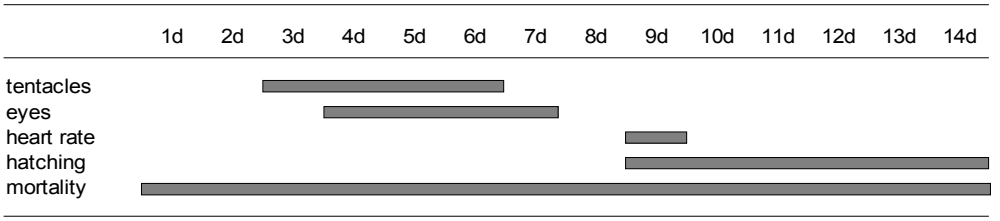
For statistical analysis we used the software JMP®4.0 (SAS). Normally distributed data (checked by Shapiro-Wilk's test) were tested for significance with Student's t-test, whereas data with non-normal distribution underwent the Kruskal Wallis test. The α-level for significant differences was set at $p \leq 0.05$.

RESULTS

Specification of endpoints

Prior to the described investigations, a series of observations under control conditions were performed to find out which endpoints could be considered to be most practicable. It is important that the formation of the observed parameters occur at first after the clearing-up of the egg. This guarantees an easy determination and a reliable evaluation. The following parameters were most feasible and should be recorded daily, or at least at the suggested days of development as shown in Tab. 1: from day four onwards, formation of the eyes; from day five onwards, formation of tentacles; and at day nine heart rate measurement. From day nine onwards, the Petri dishes should be checked for hatching, and the recently hatched snails must be weighed. Furthermore, the mortality should be recorded daily. The developmental endpoints are illustrated by Fig. 1.

Tab. 1. Schedule to investigate different parameters of embryonic development *of M. cornuarietis*



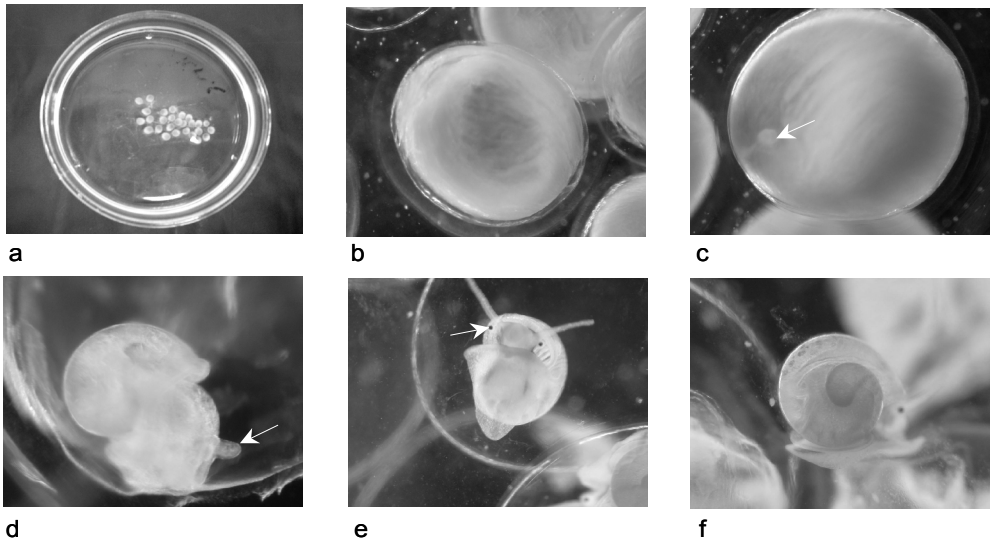


Fig.1. Developmental stages of *Marisa embryos* at different times. a: Petri dish with eggs used for embryo tests; b: Two-days-old egg, still opaque; c: partially transparent egg, embryo visible but not well differentiated (arrow); d: day five, formation of tentacle (arrow); e: day nine, snails are completely developed, eyes (arrow) are visible, and heart rate can be determined; f: snail after hatching.

Cadmium treatment

For the 500 µg/L Cd treatment, it was not possible to perform an entire embryo test. At day 12, mortality in the control group was 1.9%, and all of the survivors had hatched at this time, whereas in the exposed group the mortality was already 56% at day 9 and even 94% at day 12 (Tab. 2). Therefore, sublethal effects in the embryonic development at a concentration of 500 µg/L Cd could not be shown for *M. cornuarietis*. The following data consequently refer to the 250 µg/L Cd treatment only.

Tab. 2. Percentage of mortality in the different cadmium treatments

day	500 µg/L Cd	control	250 µg/L Cd	control
7	13	1,9	5,3	8,4
9	56	1,9	5,3	8,4
12	94	1,9	5,3	8,4
14	94	1,9	7,0	8,4

At day 7, embryos in the 250 µg/L cadmium experiment showed a trend to a delayed development in comparison to the control as symbolized by a reduced mean percentage of visible eyes and tentacles at day 7 (Fig. 2a). This finding, however, was not significant as well as the results for the heart rate at day 9, which were almost identical for the cadmium and the control group (data not shown). On the other hand, significant effects of cadmium were visible in the time of hatching. At day 12, 70% of the snails had hatched in the control and at day14 more than 90% had hatched. In the cadmium-treated group the hatching at day 12 was significantly reduced ($p \leq 0.05$) with an average of only 1.5%; and at day 14, respectively, only 3.0% had hatched ($p \leq 0.01$) (Fig. 2b). Furthermore, the weight after hatching was affected in the cadmium treatment as shown in Fig. 2c. The snails which were raised in control water did not only hatch earlier, but also had a higher mean weight than those raised in 250 µg/L Cd. Even though this reduction was not significant, it confirmed observations during the weighing procedure, where these animals appeared smaller than the controls.

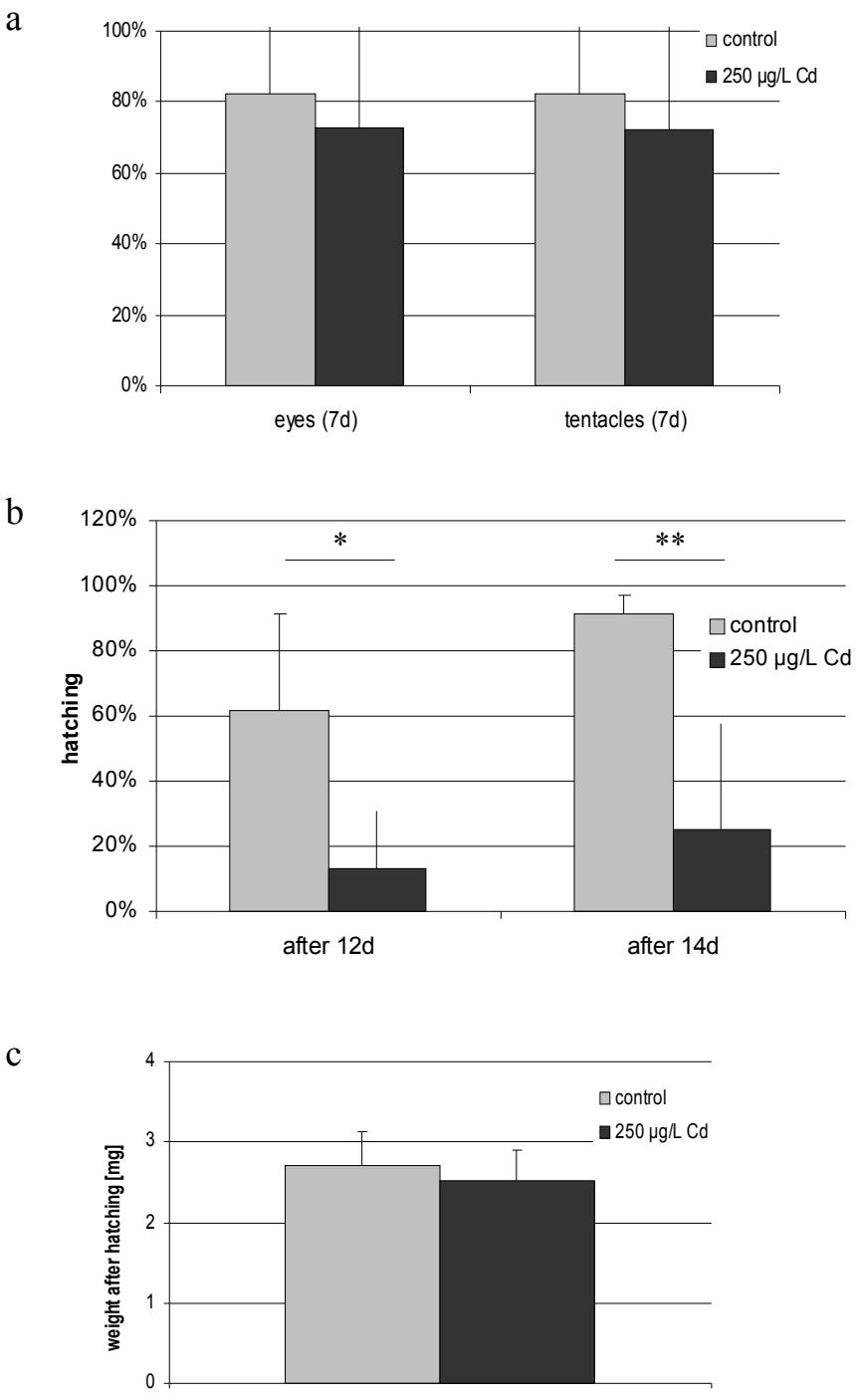


Fig.2. Water control and 250 µg/L Cd treatment, means ± SD, a: Formation of eyes and tentacles after 7 days [%]; b: Hatched snails after 12 and 14 days [%]; c: Individual weight after hatching [mg]. * significant at $p \leq 0.05$, ** significant at $p \leq 0.01$.

17α-ethinylestradiol treatment

To assess effects which may be caused by endocrine disruption, animals were raised in 10µg/L EE2. The solvent control and the water control were always tested in parallel. For the investigated parameters 'eye development' and 'tentacle development', no differences among the three groups were observed at day 7 (data not shown).

The measurement of the heat rate revealed a significant decline in the DMSO control (72 beats per minute [bpm]) compared to the water control (77 bpm). For the EE2 treatment the lowest heart beat rate was measured with only 69 bpm, which was also significant lower compared to the control group (Fig. 3a).

For the hatching, results were inconsistent between days 11 and 14 as shown in Fig. 3b. At day 11 and 13, on the average more animals hatched in the solvent control (35% respectively 90%) than in the water control (33% respectively 78%), and for both days the highest hatching rate was observed in the EE2 treatment group with 40% at day 11 and 91% at day 13. At day 12, at the average, animals raised in either DMSO or EE2 also showed higher hatching rates than the water control but, in this case, most snails hatched in the solvent control and not in the EE2 group. No differences were observed at day 14, when hatching was completed in all groups. Due to high mortality, the results were not significantly different between the groups.

The results on the weight of recently hatched animals resembled the pattern observed for the hatching rates at days 11 and 13. The average weight of animals raised in the water control was 2.8 mg, animals from the solvent control had a weight of 2.9 mg. The highest weight was measured in the EE2 group were animals had an average weight of 3.1mg (Fig. 3c). However, these results were not significantly different from one another and should therefore only be regarded tentative.

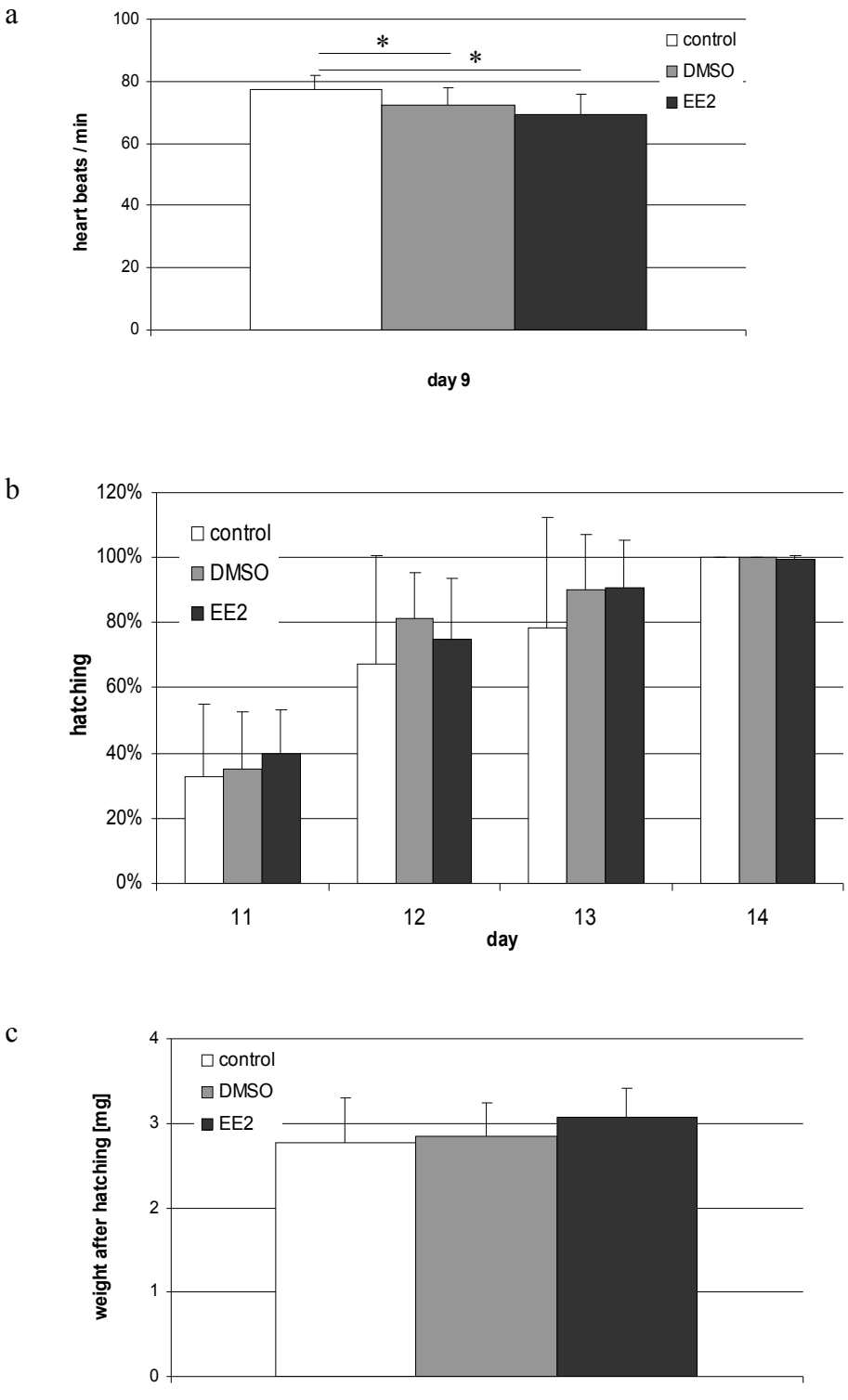


Fig.3. Water control, DMSO control, and 10 µg/L EE2 treatment, means ± SD, a: Heart rate at day 9 [min^{-1}]; b: Hatched snails between day 11 and day 14 [%]; c: Individual weight after hatching [mg]. * significant at $p \leq 0.05$.

Bisphenol A treatment

Embryos developing in the presence of 50µg/L BPA and 100µg/L BPA did not show any changes in comparison to the control group regarding the development of their eyes and tentacles. In all three treatments, almost 100% of the snails' eyes and tentacles were visible on day 7 (data not shown).

The measurement of the heart rate at day 9 revealed a significant decline from 75 beats/min in the control to 67 beats/min in animals raised in 100µg/L BPA (Fig. 4a). A similar trend was observed for hatching. At day 12 and 13 of the development, most animals had hatched in the control group followed by 50µg/L BPA, the least hatched in 100µg/L BPA (Fig. 4b). In contrast, at the beginning of the hatching at day 11, where only a small percentage of the snails (24% - 33%) had finished their development inside the egg, an opposite trend was visible which, however changed already the next day.

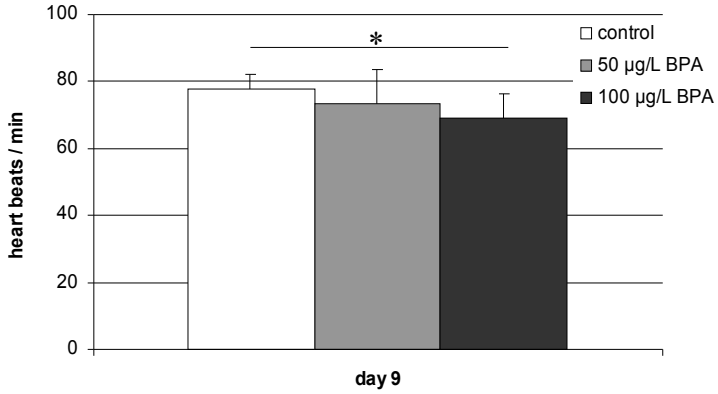
Also the weight of newly hatched individuals showed an increase with increasing BPA concentrations and was found to be significant in the 100µg/L BPA treatment vs. control (Fig. 4c).

DISCUSSION

With the establishment of an embryo test with *Marisa cornuarietis* we could successfully show its range of sensitivity that covers both lethal and sublethal toxicity. The experiment with different concentrations of cadmium demonstrated the possibility to evaluate effects on different developmental endpoints in an invertebrate, independent of mortality. In addition, we were able to select sensitive endpoints which were easy to investigate with little effort. The formation of eyes and tentacles, as well as the heart rate and hatching, well reflect the developmental status of the animals and should be investigated between days 3 and 7, according to Table 1.

The hazardous potential of cadmium and its adverse effects on a great number of organisms are well known. Mechanisms of biotoxicity and the sources of the contaminant are reviewed, for example in Pinot et al. (2000). This broad knowledge and the vast number of many studies on cadmium toxicity in several animals was the reason for the selection of this heavy metal to assess the sensitivity of our test. The observed high mortality at 500 µg/L and the sublethal effects at 250 µg/L justified our approach, since the values are in accordance to literature data.

a



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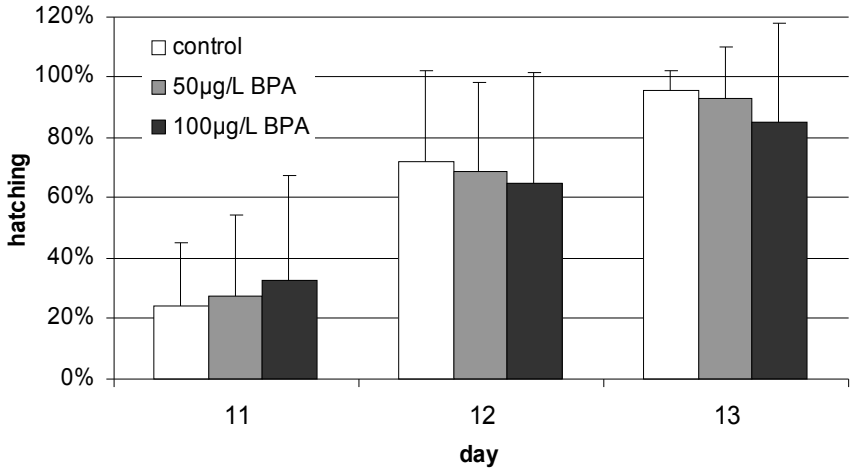

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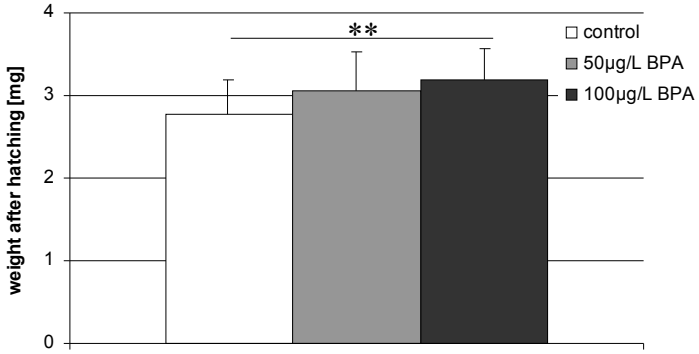

Fig.4. Water control, 50 µg/L BPA, and 100 µg/L BPA treatment, means ± SD, a: Heart rate at day 9 [min^{-1}]; b: Hatched snails between day 11 and day 13 [%]; c: Individual weight after hatching [mg]. * significant at $p \leq 0.05$, ** significant at $p \leq 0.01$.

Møller et al. (1994) reported a LC_{50} value of 1 to 4 mg/L Cd for the freshwater snail *Potamopyrgus antipodarum*, an the LC_{50} for larvae of *Salmo salar* was between 300 and 800 µg/L Cd (Rombough & Garside, 1982). In Suedel et al. (1994) different LC_{50} values for freshwater species were reported between 0.65 µg/L Cd (*Ceriodaphnia dubia*, 48h) up to 29.56 mg/L Cd (*Chironomus tentans*, 48h). In comparison to the embryo test with *Danio rerio*, the sensitivity of *M. cornuarietis* is high, as Hallare et al. (2005) reported a LC_{50} value of 30.1 mg/L Cd and no effects on the developmental rate of zebrafish at concentrations up to 10 mg/L Cd. Other studies also reported higher effective concentrations in embryo development of zebrafish than the sublethal concentrations used in our experiment (Chow and Cheng, 2003; Chan and Cheng, 2003)

In sublethal concentrations, 250 µg/L cadmium resulted in a delayed development of the embryos, reflected by delayed hatching at lower weight and the trends to delays in eye and tentacle formation. Similar effects are described for the growth of pond snails, where EC_{50} values of 58.2 and 142.2 µgCd/L were reported (Cœurdassier, 2003). Furthermore, a delay in hatching of 5 to 15 day was observed in the freshwater snail *Lymnaea stagnalis* for cadmium concentrations between 25 µg/L and 100 µg/L Cd (Gomot, 1998). Along with the delayed development of the embryos, the reduced weight after hatching supports interpreting the observed effects as symptoms of growth inhibition caused by cadmium.

Many environmental pollutants have estrogenic activity with the potential to disrupt endocrine mechanisms and developmental integrity. The effects on wild life are diverse and range from feminisation in many vertebrate species (Bergeron et al. 1994; Fry and Toone, 1981; Guillette et al., 1994) to malformations and developmental changes in invertebrates (Hutchinson, 2002; McLachlan, 2001) In this study, the exposure to 17α-ethinylestradiol showed effects on different parameters during the development of *M. cornuarietis*. The hatching rate implies a faster development of the embryos with an earlier onset of hatching in the EE2 treatment compared with the water control at days 11 to 13 (not significant). This observation is in line with further studies which reported a raised reproduction, measured for example in increased population size or egg production (Watts et al. 2002, Andersen et al. 1999). In the present case, the influence of the solvent control can not be neglected which is underlined by the significant reduction of the heart rate in both EE2 and DMSO treated snails. Similar effects caused by the regularly used solvent DMSO were previously described by Hallare (in press), and Rayburn and Fischer (1997). Regarding weight, the influence of

DMSO seemed to be lower in comparison to EE2, but no significances were observed.

Even though we knew the toxicity problem caused by DMSO, we decided to perform our test with this solvent, since previous experiments revealed an intensive growth of fungi, whenever acetone or ethanol has been used as solvents. Fungal growth was shown to influenced the embryonic development to a greater extend than DMSO in the experimental onsets (A. Bohlen and M. Schirling, unpublished).

Effects of endocrine acting substances on the apple snail *Marisa cornuarietis* are described in previous studies by Oehlmann et al. (2000) and Schulte-Oehlmann et al. (2000). In these experiments, the endpoints were assessed in adult or juvenile snails but not in embryos. For the BPA treated animals in our study, we found similarities to the EE2 experiment in the pattern of effects on the parameters heat rate and weight. Both substances induced a same effect, a decrease in the heart rate and an increase in weight measured after hatching. These changes were significant for the 100 µg/L BPA treatment in comparison to the water control. In *M. cornuarietis* as well as in other species, substances with estrogenic activity stimulated the egg production or the population size (Oehlmann et al. 2000, Giesy et al. 2000, Andersen et al. 1999). This stimulation is reflected by an increased weight of hatched animals, induced by (xeno) estrogens, as shown in our experiment.

With this study we present a first protocol for an embryo test with *Marisa cornuarietis*. This protocol includes endpoints which are easy to determine and relevant for the development of the snails. A timetable for the investigation of these endpoints guarantees the reproducibility of the test. The procedure is simple and can be performed within a short time with little expense in most laboratories. Our results showed the embryos to be sensitive to indicate the toxic potential of different chemical compounds, in concentrations which are equal or even lower than the LOECs reported for systems, e.g. the *Danio rerio* embryo test. For a more detailed protocol, the number of endpoints and the interval of investigation may be expanded to obtain further information. A clear estimation of the endocrine disrupting potential of BPA and EE2 for *M. cornuarietis* embryos by this study remains difficult. Even though we could not find significant differences on the hatching we were able to detect effects on the heart rate and weight of the snails. Therefore, we regard our results to be valuable for subsequent studies, and the embryo test with Marisa to be a promising tool in aquatic toxicology.

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