Reproductive disorders in sea urchins (*Psammechinus miliaris*) caused by environmental pollutants

by

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A thesis submitted in partial fulfilment
of the requirements for the degree of

Doctor of Philosophy
in Cell Biology

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Date of Defence, 14th of September 2009

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Contents

Abstract

Environmental contaminants are suspected to impair reproductive health of exposed organisms. Since reproductive health is essential for population sustainability and survival detailed knowledge of the detrimental effects of chemicals on processes related to reproduction is required. In aquatic environments, reproductive impairment by a variety of xenobiotics has frequently been documented in fish while investigations with marine invertebrates, particularly with sea urchins, are rare. This is surprising, since sea urchins, belonging to the group of echinoderms, are not only sentinels for marine toxicity testing but their fertilization as well as their gonadal biology are well described.

In the present study, the effects of the polycyclic aromatic hydrocarbon (PAH) phenanthrene on reproductive tissue of the North Atlantic sea urchin *Psammechinus miliaris* were investigated following maternal exposure. Female *P. miliaris* exposed to a high dose (500 µg L^{-1}) of phenanthrene for 20 days showed severe gonadal lesions. These alterations are, in general, similar to histopathological changes in other invertebrates as well as in fish following exposure to PAHs or oil. While increased atresia of previtellogenic oocytes was observed following phenanthrene exposure no effects on vitellogenic oocytes and mature ova were detected, using light microscopy. Histochemical detection of the lysosomal enzyme acid phosphatase in conjunction with computer-assisted image analyses, however, indicates that the structure of lysosomes in vitellogenic oocytes and mature eggs is altered. The effects of high (150 µg L^{-1}) and environmentally relevant (5 µg L^{-1}) concentrations of phenanthrene were also investigated on reproductive tissue of both sexes of sea urchins (*P. miliaris*). Though behaviour of sea urchins was severely affected following exposure to 150 µg L^{-1} phenanthrene for 10 days, both toxin concentrations resulted in either no or only minor changes of histological and biochemical parameters in the gonads. But, profound sex-specific differences were found in all treatments indicating that males are much more susceptible to oxidative stress than females. Measures of the energy metabolism (energy charge, AMP/ATP ratio, adenylate concentraions) further reflect the predominantly anaerobic metabolism of sea urchin gonads which is discussed as protective mechanisms for female and male gametes.

To differentiate between pollutant responses and natural variations, a population of *P. miliaris* in the northern Wadden Sea was sampled over an annual reproductive cycle and processed for light microscopy. Sex, gonadal stage and spawning were shown to affect gonadal histology and histochemistry and are, therefore, recommended to be assessed critically for

identification of xenobiotically induced changes in reproductive tissues. Further suggestions for histopathological diagnosis of sea urchin gonads, such as the application of a consistent nomenclature, are presented.

Phenanthrene as well as various other chemicals is known to interfere with cellular calcium signalling. One of the best studied Ca^{2+} signals and essential for successful reproduction is the Ca^{2+} wave at fertilization, a transient increase in intracellular Ca^{2+}, spreading the egg from the point of sperm fusion to the antipode. For the first time, the Ca^{2+} signal at fertilization was recorded using the Ca^{2+} indicator dye Fura-2 acetoxymethylester (AM) in combination with MK571, an inhibitor of multidrug resistance associated proteins. The metal copper and the brominated compound 2,4,6-tribromophenol (TBP) significantly increased the fertilization Ca^{2+} signal in *P. miliaris* at 6.3 µM and 100 µM, respectively, which might contribute to the observed reduced fertilization rates following exposure to these compounds.

The shown histopathological and cellular investigations provide a promising basis for future studies on pollutant-induced reproductive disorders not only in sea urchins but also in other invertebrates. Gonadal histopathology, for example, may be applied in biological effect monitoring while the effects of chemicals on Ca^{2+} signalling during fertilization opens up new perspectives for fundamental cell biological research.

List of selected abbreviations

ALDH	aldehyde dehydrogenase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ASW	artificial sea water
ATP	adenosine triphosphate
BPA	bisphenol A
CYP450	cytochrome P450
Δ ratio	(F340 / F380 $_{\text{at fertilization}}$) - (F340 / F380 $_{\text{prior to fertilization}}$)
EC	energy charge
F340	fluorescence intensity at 340 nm
F380	fluorescence intensity at 380 nm
G6PDH	glucose-6 phosphate dehydrogenase
GI	gonad index
GP2	general purpose medium 2
GSH	glutathione
GV	germinal vesicle
HTD	horizontal test diameter
MO	mercury orange
MRP	multidrug resistance associated protein
MYP	major yolk protein
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NADH	reduced nicotinamide adenine dinucleotide
NP	nutritive phagocyte
PAH	polycyclic aromatic hydrocarbon
PAS+	Periodic acid-Schiff positive
phe	phenanthrene
TBP	2,4,6-tribromophenol
VTD	vertical test diameter

Publications

Schäfer, S., Bickmeyer, U., Köhler, A., 2009. Measuring of Ca^{2+}-signalling at fertilization in the sea urchin *Psammechinus miliaris* and alterations of this Ca^{2+}-signal by copper and 2,4,6-tribromophenol. *Comparative Biochemistry and Physioloy C* 150: 261-269.

Schäfer, S., Köhler, A., 2009. Gonadal lesions of female sea urchin (*Psammechinus miliaris*) after exposure to the polycyclic aromatic hydrocarbon phenanthrene. *Marine Environmental Research* 68: 128-136.

Schäfer, S., Weihe, E., Abele, D., Köhler, A., *in preparation.* Sex-specific physiological response and histological changes in gonads of sea urchins (*Psammechinus miliaris*) after exposure to phenanthrene.

Schäfer, S., Köhler, A., *in preparation*. Gonadal histochemistry and histology of sea urchins (*Psammechinus miliaris*): effects of sex, reproductive stage and spawning.

Schäfer, S., Köhler, A., *in preparation*. Gonadal histopathology of sea urchins: its application in toxicology.

Schäfer, S., Köhler, A., *in preparation.* Germinoma in the sea urchin (*Psammechinus miliaris*) in the northern Wadden Sea.

Talks

Schäfer, S., Köhler, A., 2009. Gonadal histopathology of sea urchins: its application in toxicology. 15th International symposium on *Pollutant Responses of Marine Organisms* (PRIMO), 17 – 20 May, Bordeaux, France.

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Schäfer, S., Köhler, A., 2009. The polycyclic aromatic hydrocarbon phenanthrene severely affects the ovarian tissue of sea urchins (*Psammechinus miliaris*). Poster & poster spotlight. *SETAC* Europe 19th Annual Meeting, 31 May - 4 June, Göteborg, Sweden.

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Schäfer, S., Köhler, A., 2009. The effects of the heavy metal lead on the ovarian tissue of sea urchins (*Psammechinus miliaris*). Poster and poster spotlight. 15th International symposium on *Pollutant Responses of Marine Organsims* (PRIMO), 17 – 20 May, Bordeaux, France.

Schäfer, S., Köhler, A., 2008. Histopathological alteration in reproductive tissue of female sea urchin (*Psammechinus miliaris*) after exposure to the polycyclic aromatic hydrocarbon phenanthrene. Poster. *SEASINK* Conference, 26 - 28 June, Universidade Fernando Pessoa, Porto, Portugal.

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Contribution of authors and coauthors to publications

Chapter III: Gonadal histochemistry and histology of sea urchins (*Psammechinus miliaris*): effects of sex, reproductive stage and spawning

Schäfer, S., Köhler, A., *in preparation*.

The concept and design of this study was developed by myself in cooperation with AK. I took the samples, processed and measured them. I analysed the results and discussed them with AK. I wrote the manuscript which was revised together with AK.

Chapter IV: Gonadal lesions of female sea urchin (*Psammechinus miliaris*) after exposure to the polycyclic aromatic hydrocarbon phenanthrene

Schäfer, S., Köhler, A., *published in* Marine Environmental Research (2009) 68: 128-136.

I developed the concept and design of this experiment, supported by AK. I conducted the laboratory exposures, took the samples, processed and evaluated them. I analysed the results and discussed them with AK. I wrote the manuscript which was proof-read by AK.

Chapter V: Histochemical alterations in gonads of female sea urchin *Psammechinus miliaris* after exposure to the polycyclic aromatic hydrocarbon phenanthrene

Schäfer, S., Broeg, K., Köhler, A., *in preparation.*

The experiment was planned by myself in agreement with AK. I carried out the laboratory exposures, took the samples, processed and evaluated them. KB help me with the image analysis system. I analysed the results and discussed them with AK and KB. I wrote the manuscript which was revised together with AK and KB.

Chapter VI: Sex-specific biochemical responses and histological changes in gonads of sea urchins (*Psammechinus miliaris*) after exposure to phenanthrene

Schäfer, S., Abele, D., Weihe, E., Köhler, A. *in preparation*.

Together with AK, I elaborated the concept of this study. I carried out the experiments and measured the samples. The measurements with the HPLC were supported by EW. After discussing the results with AK and DA I wrote the manuscript. The manuscript was proof-read by AK, DA and EW.

Chapter VII: Gonadal histopathology of sea urchins: its application in toxicology

Schäfer, S., Köhler, A., *in preparation.*

This chapter does not involve experiments and laboratory measurements. I had the idea for this manuscript due to my previous work on gonadal histopathology of sea urchins. I discussed the concept for this manuscript with AK and revised it together with her.

Chapter VIII: Measuring of Ca^{2+}-signalling at fertilization in the sea urchin *Psammechinus miliaris*: Alterations of this Ca^{2+}-signal by copper and 2,4,6-tribromophenol

Schäfer, S., Bickmeyer, U., Köhler, A., *published in* Comparative Biochemistry and Physioloy C (2009) 150: 261-269.

I planned the experiments in agreement with UB and AK. UB introduced me to the measurement of Ca^{2+} signals. I conducted the experiments and did the measurements. I discussed the results with AK and UB. The manuscript was revised together with AK and UB.

General introduction

For the survival and long term sustainability of a population successful reproduction is a crucial prerequisite. However, reproductive health is suspected to be disturbed by many natural and man-made chemicals which end up in the marine environment. Reproductive impairment by a variety of xenobiotics have been extensively reported in fish (Mills and Chichester 2005). In marine invertebrates, the effects of chemicals resulting in imposex and abnormal sexual development have been studied in detail in molluscs (Hutchinson 2002). Nonetheless, the effects of pollution on reproduction of other invertebrate species have less often been in focus.

Reproductive health can be assessed, for example, by gonad structure and development (Mills and Chichester 2005) as well as by fertilization success (Hose 1985; Unknown 1993). In fish, gonadal histopathology has frequently been applied for the detection of potentially adverse effects of chemicals (e.g. Dietrich et al. 2009). However, the mechanisms leading to reproductive disorders in adult marine invertebrates are hardly known, though exposure of adult organisms during active gametogenesis may decrease the quality and quantity of gametes (Nacci et al. 2000; Au et al. 2001). Moreover, while fertilization success is often set as an endpoint in toxicity tests for evaluating adverse effects of environmental contaminants (e.g. Hose 1985; Caldwell et al. 2002; Hollows et al. 2007), the effects of chemicals on the complex signalling cascades involved in fertilization are not yet understood. Both – alterations of gamete production in adults as well as changes in cellular signalling during fertilization - may provide early warning signals for the detrimental effects of contaminants on reproductive function. In addition, they may help to understand the mechanisms by which chemicals impair reproductive success of marine invertebrates. This is essential for a sound risk assessment of environmental pollutants as reproduction links effects from the individual to the population level (Attrill and Depledge 1997).

Sea urchins as model organisms

Echinoderms are not only considered as valuable test species in marine toxicology but they are also popular models in cell biological research. Sugni et al. (2007) summarized the strategic advantages for the use of echinoderms in toxicological studies: Echinoderms are of major importance for marine ecosystems contributing to more than 90% of benthic biomass in the hadal and bathyal and often control benthic communities. Due to their extended external epithelia and

their benthic habits they are sensitive targets of environmental pollutants, especially for chemicals bound to and accumulating in sediments. As predators they are further susceptible to biomagnification.

Moreover, sea urchins, in particular, have long been models in developmental and cell biological research. Their developmental biology is well described which has lead to the establishment of routine fertilization and embryo development assays for assessing the toxicity of polluted marine waters as well as specific chemicals (Kobayashi et al. 1971; Hose 1985; Manzo 2004). This is primarily due to the fact that not only large number of gametes can easily be obtained from a small number of adults but that *in vitro* fertilization and embryo development can also easily be accomplished in the laboratory. In addition, sea urchins and humans both belong to the large group of deuterostomes. This key phylogenetic position gave rise to the sea urchin genome project. The genome of the sea urchin *Strongylocentrotus purpuratus*, published in 2006, is of great importance as it is the first full sequence of a non-chordate deuterostome (Davidson 2006). Goldstone et al. (2006) surveyed this genome for homologs of gene families involved in the protection against chemical stressors. They identified more than 400 chemical defence genes including e.g. cytochrome P450 and ATP-dependent efflux transporters genes.

Finally, some sea urchin species are of socio-economical relevance as their reproductive tissues are edible. Harvesting of this so-called roe resulted in overexploitation of natural sea urchin stocks in many regions and led to commercial culture of several sea urchin species.

The biology of the sea urchin *Psammechinus miliaris*

As test species for the present study we chose the sea urchin *Psammechinus miliaris* (Echinodermata, Echinoida) (Figure 2) which is abundant in the northern Wadden Sea. The gametes and embryos of this species have previously been used in toxicity test in our laboratory (Hayden 2005, Köhler 2002) as well as by other scientists (Klöckner et al. 1985; Caldwell et al. 2002; Caldwell et al. 2004; Anselmo et al. 2009). *P. miliaris* is a relatively small sea urchin whose horizontal test diameter usually does not exceed 40 mm (Kelly and Cook 2001). It is found in the North Atlantic, from Scandinavia (North) to Morocco (South) but is absent from the Mediterranean Sea (Kelly and Cook 2001). In the North Sea its distribution is confined, in particular, to the Dogger Bank, the German and the Danish Wadden Sea as well as the Southern Bight (Kelly and Cook 2001).

P. miliaris is often greenish in colour with violet tips at the spines (Kelly and Cook 2001; Jackson 2008). Its depth range extends from approximately 100 meter depth to the littoral zone (Campbell 1977). Intertidally *P. miliaris* is found on rocky shores and seaweeds, whereas subtidally it occurs in seagrass beds or on mixed coarse bottoms (Jackson 2008). It is well documented that *P. miliaris* is omnivorous whereby gonad biochemistry indicate higher levels of carnivorism in intertidal individuals compared to subtidal populations (Hughes et al. 2005).

Figure 2: The sea urchin *Psammechinus miliaris* (Foto: A. Hayden, 2005).

In the present study, *P. miliaris* were obtained from the List tidal Basin (Germany) in the 'Königshafen' which is part of the Sylt Rømø Bay in the northern Wadden Sea. In the 1930s, Wohlenberg (1937) found *P. miliaris* in the 'Königshafen' only below the middle low water line in beds of the sea grass *Zostera marina* or in deeper waters. However, recent studies on sea urchin populations in this area are missing. *P. miliaris* has been shown to be robust in culture (Kelly 2002) and can be routinely raised from the larval phase in the hatchery (Kelly et al. 2000). Moreover, due to its pleasantly flavoured roe Scottish scientists have investigated the potential of this species for aquaculture during the past decade (Cook et al. 1998; Kelly et al. 1998; Symonds et al. 2009).

Gametogenesis and fertilization – potential targets for toxic action

Like most other sea urchin species *P. miliaris* has an annual cycle of gametogenesis with one spawning period (Kelly 2000). In both sexes, the gametes develop in the gonads which are located aborally in the test. Due to their pentamer symmetry, sea urchins possess five gonads with separate gonadopores (Westheide and Rieger 1996) which can be used for sex determination in *P. miliaris* (Uhlig 1979). The gonads are large rebranched lobes ending in blind acini which are surrounded by a multi-layered acinal wall (Palmer Wilson 1940; Chatlynne 1969). Germ cells are generated at the inner epithelial layer of the acinal wall and migrate towards the lumen of the acinus during maturation. Next to the germ cells, accessory cells, the nutritive phagocytes (NP), are present in the gonads which are supposed to deliver nutrients to the gametogenic cells during maturation (Walker et al. 1998).

During gametogenesis, gametes develop from primordial germ cells which become oogonia and spermatogonia by arriving in the reproductive tissue during organogenesis. In the gonads fertile, haploid ova and spermatozoa are finally derived from the oogonia and spermatogonia, respectively (Figure 3). Thereby, the diploid oogonia and spermatogonia proliferate mitotically to form secondary oogonia and spermatogonia which enter into the first meiotic division and become oocytes and spermatocytes, respectively. During meiosis, the genetic material is first replicated by duplicating each of its chromosomes. Then the homologous chromosomes are segregated and the sister chromatids are split during the first and second meiotic divisions, respectively (Alberts et al. 2002). In males, symmetric divisions yield four equal-sized gametes during spermatogenesis. In contrast, asymmetric divions of sea urchin oocytes during meiosis I and II form a single egg and two unequal polar bodies (Song et al. 2006, Figure 3). Next to nuclear maturation, gametogenesis includes cytoplasmic changes such as alterations in organelles, mRNA and protein patterns as well as physiological performance. Sea urchins belong to a limited group of organisms, wherein an oocyte completes meiotic maturation forming a haploid egg before it becomes fertile (Voronina et al. 2003).

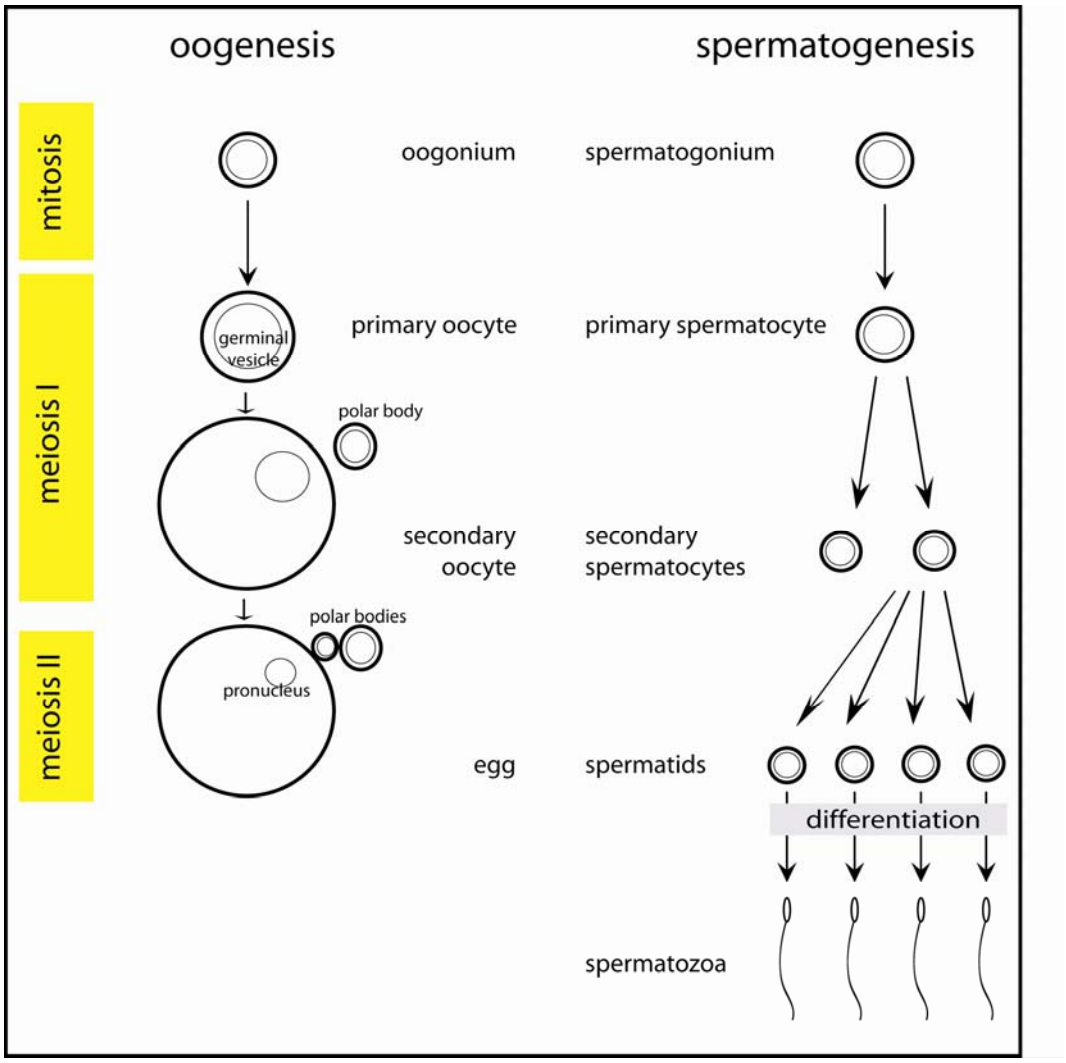

Figure 3: The stages of gametogenesis with oogenesis (left) and spermatogenesis (right). Oogonia derive from primordial germ cells that migrate into the developing gonad early in embryogenesis. After a number of mitotic divisions, oogonia begin the first meiotic division, after which they are called primary oocytes. Completing meiotic division I, they become secondary oocytes which eventually undergo meiotic division II to become mature ova. During gametogenesis the nucleus forms the so called germinal vesicle which breaks down before generating the egg. Likewise, spermatogonia are generated by primordial germ cells and divide mitotically to form primary spermatocytes. Primary spermatocytes become secondary spermatocytes and spermatids after meiotic division I and II, respectively. The haploid spermatids differentiate into fertile spermatozoa. Note that division of a primary oocyte results one mature egg while from each primary spermatocyte four spermatozoa are formed. Furthermore, female germ cells increase while male germ cells decrease in size during maturation (Figure modified after Alberts et al. (2002) and Song et al. (2006)).

Being broadcast spawning invertebrates, sea urchins directly release huge numbers of gametes in the water column where fertilization occurs and embryo-larval development proceeds. The availability of many gametes as well as the external insemination have led to intensive research of the cellular and molecular events at fertilization in sea urchins. On binding of the sperm to the egg, a cascade of events is initiated by a local increase in cytosolic Ca^{2+} spreading the egg in a

wave-like fashion (Figure 4). This calcium wave, a universal and ubiquitous event during fertilization in all organisms, has been termed the 'beginning of life' (Santella et al. 2004).

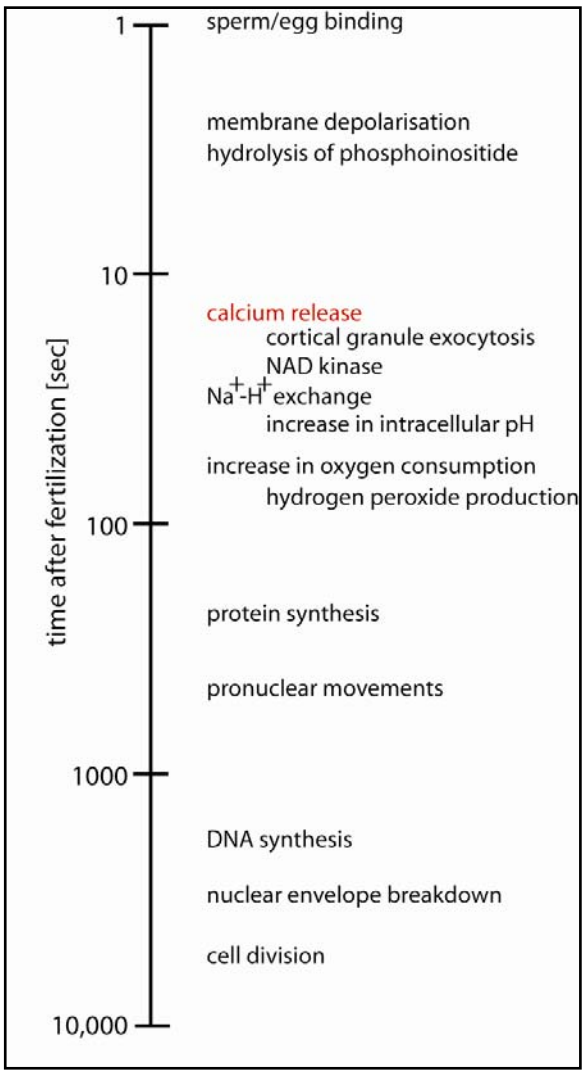

Figure 4: The sequence of changes initiated at fertilization in the sea urchin embryo. Timing is logarithmic and indicates the approximate timing of changes in embryos of the sea urchin *Strongylocentrotus purpuratus* at 17°C. (modified after Epel (1990)).

In sea urchins the Ca^{2+} is released from intracellular stores probably by formation of inositol-1,4,5-triphosphate ($InsP_3$) while nicotinic acid adenine dinucleotide phosphate (NAADP) as well as cyclic ADP ribose (cADP) are also supposed to be involved (Steinhardt et al. 1977; Santella et al. 2004; Whitaker 2006). Early during fertilization, Ca^{2+}-mediates the exocytosis of the cortical granules which release their contents into the perivitellinic space underneath the vitelline coat, resulting in elevation and modelling of the fertilization membrane. This creates a permanent block to polyspermy, the fusion of more than one sperm, which would be lethal for the egg. Calcium

further activates various enzymes such as NAD kinase and glucose-6-phosphate dehydrogenase. NAD kinase catalyzes the conversion of NAD into NADP and NADPH. NADPH is essential for producing hydrogen peroxide for hardening of the fertilization membrane (Epel 1990). Another early post-fertilization event, mediated either by protein kinase C or directly by elevated calcium is the activation of the Na^+-H^+-exchange elevating the intracellular pH (Epel 1990). The pH change probably initiates the increased cellular respiration and protein synthesis within a few minutes after insemination. After around 20 minutes the male pronucleus finally fuses with the female pronucleus (karyogamy) generating the diploid nucleus of the zygote.

Environmental pollutants

For the present thesis we selected a number of chemicals for experimental exposure studies which have high relevance for the marine environment due to their ubiquitous presence and their toxicity. Lipophilic organic compounds such as polycyclic aromatic hydrocarbons (PAHs) are widespread contaminants in the environment. PAHs are considered as hazardous substances by several European and regional directives aiming at protecting aquatic environments from anthropogenic impact (HELCOM; OSPAR 2007; EC 2008a). A major component of total PAHs in the marine environment is phenanthrene, a low molecular weight, 3-ring polyaromatic hydrocarbon (Figure 1, Law et al. 1997). Phenanthrene, also classified as priority pollutant by the United States Environmental Protection Agency (ATSDR 1990), comprises an important petroleum-source (petrogenic) PAH usually found in higher amounts in refined oil products than in crude oil. Petroleum products including e.g. kerosene, gasoline and diesel fuel are emitted directly to marine environments through oil spills and routine tanker operations. Additionally, petrogenic PAHs may also reach the environment by natural oil seepage (Boonyatumanond et al. 2006).

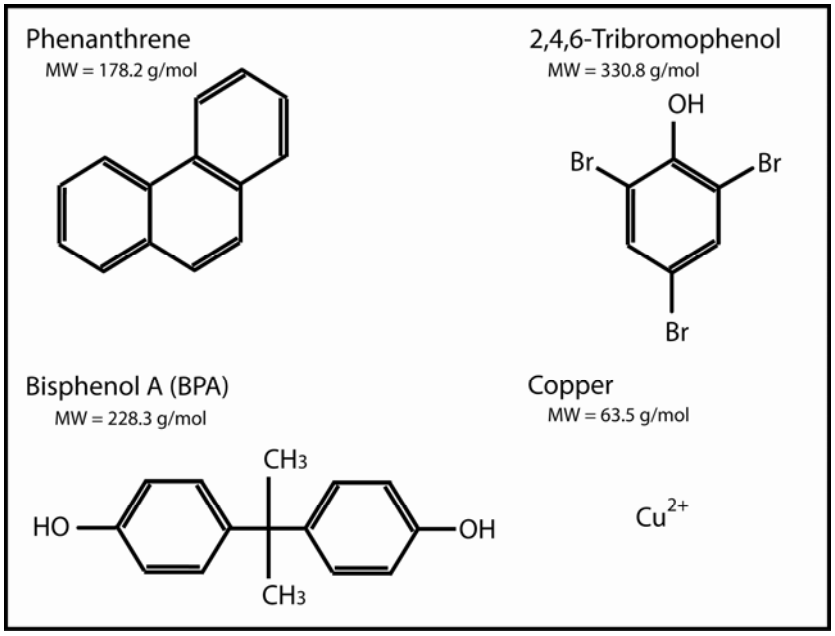

Figure 1: Structure and molecular weight of chemicals used in the present study.

Another organic pollutant of marine waters is bisphenol A (BPA, syn. 4,4'-isopropylidenediphenol, 2,2-bis(4-hydroxyphenyl)propane, Figure 1), an important key monomer in the production of polycarbonate plastics and epoxy resins as well as a non-polymer additive to other plastics. Due to incomplete polymerisation as well as degradation of the polymers BPA leaches out of the plastic. To the aquatic environment BPA is constantly released through various direct and indirect sources such as BPA production and effluent from sewage treatment plants (EC 2008; Kashiwagi et al. 2009). BPA and related compounds are common pollutants of freshwater and seawater ecosystems resulting in chronic exposure of humans and wildlife. They have been shown to disrupt normal action of endogenous hormones, leading to changes in hormone-mediated responses (Kashiwagi et al. 2009). BPA is a known xenoestrogen - a group of substances that evoke estrogenic responses (Watson et al. 2007). However, it can also affect the androgen system, disrupt thyroid hormone function and alter developmental processes (Wetherill et al. 2007). Currently, the European Communities (2008b) stated that for bisphenol A exposure of marine organisms more detailed risk assessment need to be carried out.

Of primary concern for marine life are also metals. They are not only major constituents of industrial waters but they are also persistent in the environment, move up the food chain and cause several disorders. Currently, anthropogenic inputs of metals to the sea exceed natural inputs and a large part of metals finally accumulates in the sediment (Daby 2006 and studies cited

therein). Copper, though an essential metal for all eukaryotic organisms, can reach toxic levels in aquatic environments (Bryan and Langston 1992; Stohs and Bagchi 1995; Zorita et al. 2006). Sources of copper contamination in the marine environment vary and include discharges from copper mines as well as leakage of copper from copper-based antifouling paints on ships (Zorita et al. 2006; Leon and Warnken 2008). During exposure to copper hydrochinone is suspected to be involved in the formation of reactive oxygen species causing cytotoxicity as well as DNA damage (Stohs and Bagchi 1995).

In contrast to the previously mentioned compounds, brominated phenols are mainly derived from production by benthic organisms in the marine environment (Howe et al. 2005). High concentrations of bromophenols have been recorded in algae, marine invertebrates as well as in fish (Whitfield et al. 1999). The most abundant isomer is 2,4,6-tribromophenol (TBP) which is also an industrially produced flame retardant intermediate for the production of epoxy resin as well as a wood preservatives. Bromophenols have further been identified in emissions of leaded petrol and as by-products of desinfection during food processing and water treatment using bromine. Currently, it is unkown if bromophenols may leach from plastics containing fire retardants derived from brominated phenols. However, bromophenols are not readily biodegradable and are supposed to persist in the environment as long as they are not degraded by specialized microorganisms or natural communities. Furthermore, they have a moderate to high bioconcentration potential and are suspected to bioaccumulate in marine food chains (Boyle et al. 1992; Howe et al. 2005).

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Scope of the thesis and objectives of the study

The aim of the present study is to investigate the effects of chemicals on reproductive tissue as well as on fertilization in the sea urchin *Psammechinus miliaris*. This knowledge is essential to gain insights into the mechanisms resulting in impaired reproduction in marine invertebrates.

For this purpose, mainly microscopic techniques (light and fluorescence microscopy) were applied in order to detect cellular and tissue alterations in gonads of sea urchins and to record changes in signalling events during fertilization in sea urchin eggs. At first, monthly samples of the reference population of *P. miliaris* were analysed histologically and histochemically over an annual reproductive cycle to differentiate between pollutant-induced changes and responses to natural modulators (Chapter III). The effects of the polycyclic aromatic hydrocarbon phenanthrene on the ovarian tissue of *P. miliaris* were investigated and morphological and cellular alterations were described in detail using light microscopy (Chapter IV). Consequently, histochemical techniques were applied to measure responses of, for example, potential subcellular targets (lysosomes) and detoxifying enzymes (glucose-6-phosphate dehydrogenase) directly within oocytes and eggs (Chapter IV). In order to investigate different susceptibilities of female and male sea urchins towards environmentally relevant concentrations of phenanthrene histological indices in the gonads were combined with biochemical analyses of the reproductive tissue (Chapter VI). In conclusion of the previous investigations, the use of sea urchin gonadal histopathology in toxicology is summarized (Chapter VII).

In a different set of experiments, the potential involvement of Ca^{2+}-signalling in the toxicity of different chemicals (copper, 2,4,6-tribromophenol, phenanthrene, bisphenol A), which are known to disturb Ca^{2+}-signalling in various cell types, were investigated in sea urchin eggs. For measuring changes in intracellular levels of Ca^{2+} in eggs the fluorescent Ca^{2+} indicator dye Fura-2 acetoxymethylester (AM) and the MRP inhibitor MK571 were applied (Chapter VIII). The discussion summarizes the outcome of the experiments and opens up new perspectives for future studies.

The following questions were addressed in particular:

- Do gonadal stage, sex as well as spawning affect histological and histochemical parameters in sea urchins and potentially confound pollutant-mediated responses?
- What are the cellular targets for the toxic action and what kind of histopathological effects can be found in gonads of sea urchins following phenanthrene exposure?
- Can alterations of lysosomal structures and changes in enzyme activities as well as in glutathione levels indicate early detrimental effects in oocytes and eggs following maternal exposure to phenanthrene?
- How does the energy metabolism (energy charge, sum adenylates, AMP/ATP ratio) as well as the ascorbate levels in the gonads respond to phenanthrene exposure?
- Can histological and biochemical parameters in the gonads hint at different susceptibilities of female and male sea urchins to phenanthrene exposure?
- What needs to be considered when using sea urchin reproductive tissue in toxicological research?
- Do chemicals which are known to disturb Ca^{2+} homoestasis affect the Ca^{2+}-signalling at fertilization in sea urchins?

The approaches taken to address these questions were the following:

- Laboratory studies were carried out with adult sea urchins which were exposed to phenanthrene – Chapter IV, V, VI;
- Histological and histochemical alterations in the gonads were analysed over an annual reproductive cycle in the reference population from the northern Wadden Sea – Chapter III;
- Histopathological and histochemical responses to phenanthrene exposure in the reproductive tissue were described and measured using light microscopy – Chapter IV, V, VI;
- Effects of phenanthrene exposure on biochemical parameters in the gonads were studied – Chapter VI;

- Considerations and recommendations for the use of gonadal histopathology of sea urchins for toxicology are summarized – Chapter VII;
- The toxicity of different chemicals (copper, TBP, bisphenol A, phenanthrene) on fertilization success were assessed and alterations of Ca^{2+} signals after and during chemical exposure (copper, TBP) were recorded by applying a novel methodology – Chapter VIII.

Gonadal histochemistry and histology of sea urchins (*Psammechinus miliaris*): effects of sex, reproductive stage and spawning

Schäfer, S., Köhler, A., *in preparation*.

Abstract

For the evaluation of contaminant-induced histopathological changes in gonads of sea urchins detailed knowledge about the natural variability and sex-specific differences of the tissue is essential. Moreover, for biomonitoring studies and toxicological experiments it has to be considered that suitable gametes for histological analysis are only available in specific stages of the reproductive cycle. Sea urchins (*Psammechinus miliaris)* were sampled at approximately monthly intervals from December 2006 to March 2008 in the List Tidal Basin (northern Wadden Sea). Using light microscopy, potential test organisms in the growing, premature and mature stages were predominantly found from March till June. Gonad indices (GI) showed no clear seasonal trend but correlated significantly with the reproductive stage of the animals with highest values found in growing and premature gonads of sea urchins. The amount of lipofuscin and PAS+ material were quantified in the gonads with the aid of computer-assisted image analysis. Lipofuscin content was highest in spent animals indicating that lipofuscin is accumulated during gametogenesis and eliminated during tissue regeneration. Males showed a significantly higher amount of lipofuscin than females which may be related to a lower antioxidant scavenging potential compared to females. PAS+ content in the gonads was highest during recovery and growing period and decreased during gametogenesis reflecting the storage of nutrients in the accessory cells and their consumption and packaging in developing oocytes. Other tissue alterations such as degeneration of germ cells and fibrosis of the acinal wall are described and their prevalences are discussed in relation to the seasonality of the tissue.

Key words

Psammechinus miliaris, reproductive cycle, germ cell necrosis, atresia, lipofuscin, gonad

Introduction

Histology is widely used for evaluating the health status of marine organisms (Yevich and Barszcz 1983; Stentiford et al. 2003; Au 2004). Gonadal histopathology is highly important for the early detection of reproductive disorders and for the understanding of impaired reproductive success caused by exposure towards environmental contaminants. Nevertheless, so far only few studies have focused on gonads (Blazer 2002) as these are highly dynamic tissues with seasonally varying functions and composition. The complexity and seasonality of these organs make biodiagnosis difficult and require experienced and specialized pathologists. Moreover, in sea urchins suitable gametes for the histopathological assessment of the reproductive tissues are only present in the growing, premature and mature stages (Schäfer & Köhler unpublished).

In several studies, the effects of environmental pollutants on the reproductive tissue of the green sea urchin *P. miliaris* have been investigated (Schäfer and Köhler 2009a, Schäfer et al. unpublished). This echinoid species is common in the Atlantic from Scandinavia to the Azores including the North Sea and the Baltic Sea (Boschma et al., 1932; Campbell, 1977). Sea urchins are dioecious and due to their pentaradial body organisation they possess five gonads with separate gonadopores (Westheide and Rieger 1996). The gonads are large rebranched lobes ending in blind acini which are surrounded by a multi-layered acinal wall (Palmer Wilson 1940; Chatlynne 1969). Germ cells are formed at the germinal epithelium of the acinal wall and migrate towards the lumen of the acinus during maturation (e.g. Byrne 1990; Walker et al. 1998; Kelly 2000). Next to the germ cells, accessory cells, the nutritive phagocytes (NP), are present which are supposed to deliver nutrients to the gametogenic cells during maturation and resorb unshed or degenerating germ cells at the end of the gametic cylce (Walker et al. 2005).

In most sea urchin species living at higher latitudes, gametogenesis is a single annual event with one spawning period (Czihak 1975; Walker et al. 1998). The reproductive cycle of echinoids is considered to be controlled by exogenous factors such as temperature, photoperiod, and food availability (e.g Byrne 1990; Spirlet et al. 1998; Walker and Lesser 1998; Kelly 2000; Kelly 2001; Böttger et al. 2006). At the west coast of Scotland, *P. miliaris* is reported to have a clearly defined annual reproductive cycle with a single spawning period in June and July (Kelly 2000).

Using light microscopy, several histological indices can be measured for the evaluation of toxicologically mediated adverse effects: Lipofuscin, also known as age pigment, is widely regarded as end product of protein and lipid peroxidation due to oxidative stress (Au et al. 1999; Au 2004;

Terman and Brunk 2004). Increased accumulation of lipofuscin in the digestive gland of mussels or in the liver of fish has been shown to be associated with contamination by anthropogenic pollutants (Krishnakumar et al. 1994; Krishnakumar et al. 1997; Au et al. 1999; Au 2004). In sea urchins deposition of lipofuscin in the gonads has been linked to pollution of the respective habitat (Vashchenko and Zhadan 1993; Vaschenko et al. 2001; Vashchenko et al. 2001). Though, in laboratory studies with female *P. miliaris* lipofuscin content in the ovaries was not increased after exposure to the polycyclic aromatic hydrocarbon phenanthrene (Schäfer and Köhler 2009a) or the heavy metal lead (Schäfer and Köhler 2009b). Degeneration and resorption of germ cells has been used as a marker for the assessment of pollutant effects on sea urchins (Vashchenko and Zhadan 1993; Vaschenko et al. 2001; Vashchenko et al. 2001, Schäfer and Köhler 2009) as well as in other invertebrates (e.g. Aarab et al. 2006; Ortiz-Zarragoitia and Cajaraville 2006) and fish (e.g. Au 2004; Dietrich et al. 2009). Indeed, degeneration of oocytes, so called oocyte atresia, is also a normal physiologic event which may turn into a pathologic symptom after chemical exposure (Blazer 2002; Dietrich et al. 2009). In sea urchins, not only mature but also premature individuals may release part of their gametes before reaching maturity (Spirlet et al. 1998). Since relict gametes are phagocytosed by the nutritive phagocytes after spawning (Reunov et al. 2004) spontaneous spawning events may result in early resorption of gametes.

In many animal species the accumulation of sufficient yolk to sustain embryogenesis is a key feature of oocyte development. Yolk is a mixture of carbohydrates, lipids, nucleic acids and proteins (Armant et al. 1986) with vitellogenin being the major precursor for the egg-yolk protein in most oviparous animals (Sappington and Raikhel 1998). Levels of vitellogenin and vitellogenin-like proteins have been used as a biomarker for exposure to estrogens or estrogen-mimics in fish and mussels (Denslow et al. 1999; Fossi et al. 2002; Segner et al. 2003; Aarab et al. 2004; Aarab et al. 2006; Ortiz-Zarragoitia and Cajaraville 2006). The major yolk protein (MYP) of sea urchins, however, is an iron-binding, transferrin-like glycoprotein which is, in contrast to vitellogenin, also synthesized and stored in the testes (Brooks and Wessel 2002; Unuma et al. 2003). Yolk platelets are known to be the major PAS+ staining organelle in sea urchin eggs (Immers 1960). The periodic acid Schiff's reagent (PAS) stains structures containing a high proportion of carbohydrate macromolecules such as glycogen, glycopotein and poteinglycans and has previously been used for the detection of yolk glycoproteins in sea urchins (Armant et al. 1986; Walker et al. 2005).

In the present study, histological and histochemical changes in the gonads of sea urchins (*P. miliaris*) were investigated with regard to the reproductive stage, the sex and spawning of the

animals. Lipofuscin content and the amount of PAS+ substances were quantified with the aid of computer-assisted image analysis. Moreover, potential pathological features such as oocyte atresia and sperm necrosis are described morphologically and analysed in regard to the natural variability of the tissue.

Materials and Methods

Measurement of water temperature and day length

Seawater surface temperature data for the sampling area were obtained from a long term time series in the List tidal basin (54°50 – 55°10N, 8°20 – 8°40E, northern Wadden Sea, Germany, Figure 1) (e.g. Martens and van Beusekom 2008; van Beusekom and Reise 2008) and van Beusekom (pers. communication). Briefly, the authors measured sea water temperature in one meter depth twice a week depending on weather conditions. Data are presented as monthly mean values for the sampling period from December 2006 to March 2008.

Data for day length at the sampling station were extracted from the Astronomical Applicants Department (2008) and averaged for each sampling month.

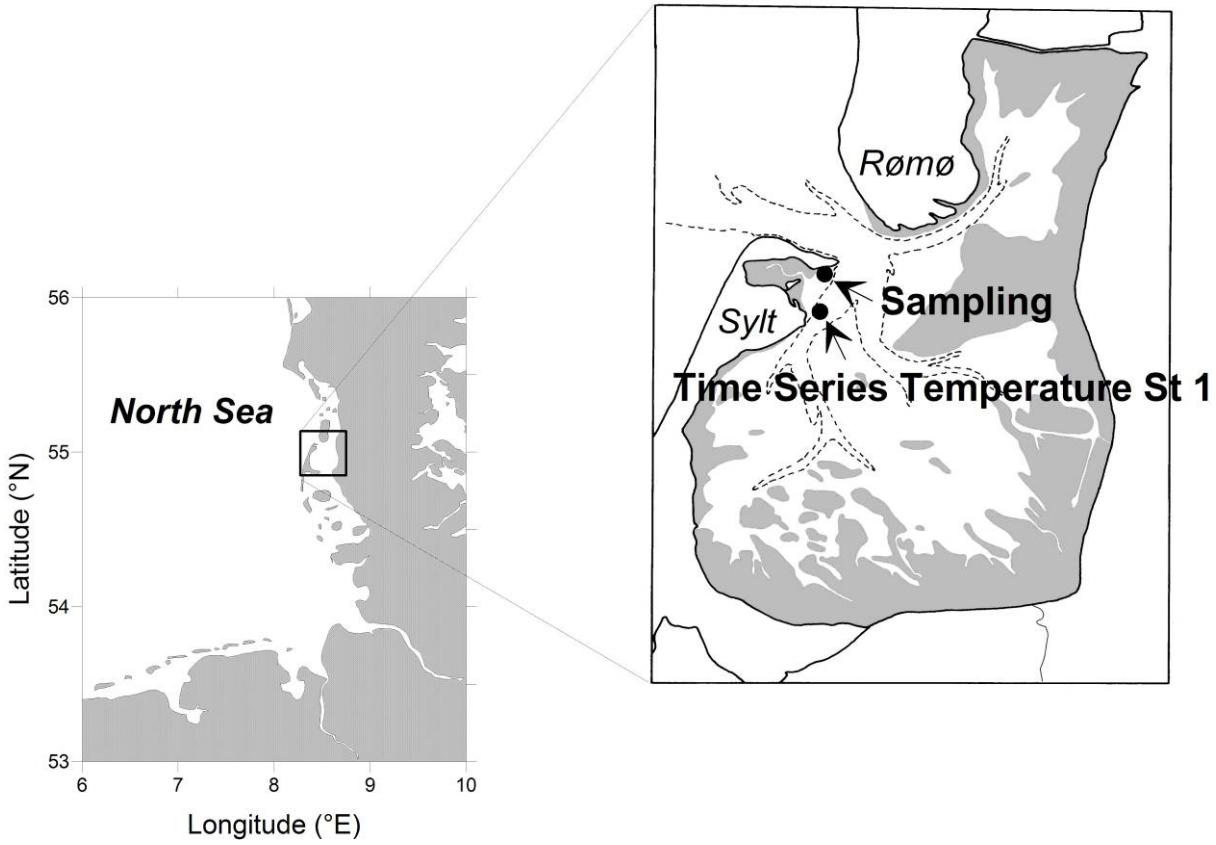

Figure 1: Map of the List Tidal Basin (situated in the northern Wadden Sea). In the insert the sampling station for the sea urchins and the position of the sampling station for the temperature time series are indicated. The extent of the tidal flats (hatched area) and the 5 m depth line (stippled line) are shown.

Sampling

P. miliaris were collected at approximately monthly intervals from December 2006 to March 2008 in the List Tidal basin (55° 02, 40 N and 08° 27, 25 E) close to the sampling station for the temperature long term time series (Figure 1). Each month 16 replicate samples (eight females and eight males) were taken. Due to logistical restrictions no samples were collected in April 2007. At the sampling site the waters are three to four meters deep with sandy bottom and a slow current (Hussel B, Wadden Sea Station Sylt (Germany), pers. communication). Samples were taken with a beam trawl, separated by sex phenotypically by the appearance of their gonadopores according to Uhlig (1979) and transported to the laboratory in Bremerhaven. Sea urchins were maintained in seawater aquaria until they were dissected (within 48 hours). Horizontal and vertical test diameters and somatic weight were measured with a calliper. Animals with a minimum horizontal test diameter of 20 mm were dissected. The gonads were removed, weighed and immediately fixed in Baker's calcium formol (4% formaldehyde and 2% calcium acetate) over night. The gonad index

(GI) was calculated as the wet weight of the gonad divided by the total somatic wet weight of the sea urchin and expressed as percentage.

Embedding, sectioning and staining

After fixation, samples were transferred in gum sucrose (30% sucrose and 1% gum arabicum) and stored at 4°C. Gonad samples were dehydrated with 70% and 100% acetone and embedded in methacrylate as described elsewhere (Köhler, 2004). After the embedding procedure, tissue blocks were left to dry for at least two days and serial sections of 2 µm thickness were cut on a microtome (HM Leica RM 2145). Sections were stained with hematoxylin & eosin (H&E) and Alcian Blue/PAS for general tissue morphology and with the Schmorl's technique for demonstration of lipofuscin as described by Schäfer and Köhler (2009a). Furthermore, for demonstration of PAS+ material sections were stained with the periodic acid Schiff's (PAS) procedure which is similar to the Alcian Blue/PAS technique but omits the counterstains with Alcian Blue and hematoxylin. This enables the exclusive demonstration of PAS+ material without staining other cellular components such as nuclei which would impede image analysis assisted quantification.

Microscopy

Sections were viewed with a microscope (Axioskop, ZEISS) and images were taken with a camera (MRc, ZEISS) coupled to a computer equipped with the software AxioVision (Vers. 4.6.3.0, ZEISS). Each individual was assigned a reproductive stage from I to VI according to Byrne (1990) and Kelly (2000) (stage I: recovery; stage II: growing; stage III: premature; stage IV: mature; stage V: partially spawned; stage VI: spent). For histological analysis, samples were analysed from December 2006 to November 2007. Due to difficulties with the embedding procedure, some methacrylate samples were lost from the samplings in August to November 2007 (August n=17, September n=12, October n=12, November n=17 replicates left).

Since sea urchins may shed part of their gametes before reaching maturity (Spirlet et al. 1998) spawning was graded in premature and mature individuals according to a scheme from 0 – 2 (0 – no spawning, 1 – minor spawning, 2 – moderate spawning). Since the animals often released gametes upon dissection of the gonads 'minor spawning' (grade 1) may be regarded as an artifact due to sampling of the tissue. Sea urchins, however, also spawned prior to sampling due to handling stress during fishing, sampling and transportation. Therefore, we can not clarify if untimely spawning had already occurred in the field.

Tissue sections stained with the Schmorl's method were assessed for lipofuscin content using computer assisted image analysis as described in detail elsewhere (Schäfer and Köhler 2009a). Briefly, black and white images were taken with the above described image analysis system. Lipofuscin was quantified in individual acini at 100x magnification with the aid of a treshold function which discriminates the darker stained lipofuscin granules from the brighter background. Similarly, PAS+ material was quantified with the same image analysis system at 100x magnification. For contrast enhancement, however, a green filter was used. In contrast to lipofuscin, PAS+ material is not only localized in granules but also laminarly which makes discrimination of the intensely magenta stained PAS+ material from the background difficult. Light settings and exposure time of the camera were, therefore, carefully calibrated prior to measuring of PAS+ material and kept constant during measurements.

Statistics

Data for gonad indices, lipofuscin and PAS+ material were BoxCox-transformed with $(GSI^{0.2} - 1) / 0.0735$, $((Lipofuscin + 1)^{-8} - 1) / -0.27578$ and $((PAS+ material + 1)^{-6} - 1)/ -0.0952$, respectively. Due to inhomogeneous variances, monthly changes in gonad indices were tested with a Kruskal-Wallis ANOVA on ranks and a Dunn's test as post hoc test. Differences between the GI of females and males were tested with a t-test. The effects of reproductive stage on gonad indices were tested with a one-way ANOVA and a Tukey HSD as post hoc test. Lipofuscin and PAS+ material may be affected by sex and reproductive stage. We tested for differences between sexes and reproductive stages by means of a two-way ANOVA. For lipofuscin and PAS+ material interactive effects between sex and reproductive stage were tested. As post-hoc test the Tukey HSD was run. Correlations were tested with the Spearman Rank Order Correlation. The effects of spawning events on sperm necrosis and oocyte atresia were analysed with a Kruskal-Wallis ANOVA on rank's and a multiple comparison procedure as post hoc test.

Results

In the following paragraph, the reproductive cycle of the sea urchin *P. miliaris* is described in detail. Thereafter, the gonad indices and the amount of lipofuscin and PAS+ material will be analysed in regard to the reproductive stage and the sex of the animals. Furthermore, cellular changes such as germ cell necrosis and fibrosis will be described.

Ovarian stages

In females, ovaries may contain many relict oocytes during the recovery period (stage I, Figure 2a). The relict oocytes undergo atresia and are resorbed by nutritive phagocytes (NPs). At the beginning of the recovery period, the NPs are visible as loose meshwork with vacuolated appearance. The NP meshwork becomes denser and accumulates PAS+/eosinophilic granules which appear magenta (Alcian Blue/PAS) and purple (H&E), respectively. At the end of the recovery period primary oocytes (10-20 μm) may be present along the acinal wall. They are stained blue with Alcian Blue/PAS as well as with H&E indicating strong basophilia. In the growing phase (stage II, Figure 2b), the oocytes increase in size (20-70 μm) and accumulate PAS+/eosinophilic material in the cytoplasm. The oocytes remain attached to the acinal wall. NPs form a dense meshwork across the acini and are filled with intensely stained PAS+ granules. Relict oocytes from the previous oogenic cycle may be present. In premature (stage III, Figure 2c) ovaries, oocytes of all developmental stages are present. Large vitellogenic oocytes stained purple with H&E and magenta with Alcian Blue/PAS start migrating towards the acinal lumen. Mature oocytes (85-110 μm) which lack the germinal vesicle accumulate in the acinal lumen. The amount of PAS+ material in the NPs is decreased. In mature (stage IV, Figure 2d) females, the lumen is filled with a large cohort of ripe ova. Few previtellogenic and vitellogenic oocytes are present close to the acinal wall. NPs are either absent or form a thin, pale meshwork around the smaller oocytes. PAS+ material in the acini is reduced or absent. During the partially spawned stage (stage V, Figure 2e), spaces appear which had been vacated by shed oocytes. In spent ovaries (stage VI, Figure 2f), acini appear empty except for relict oocytes. NPs may be evident as loose meshwork close to the acinal wall.

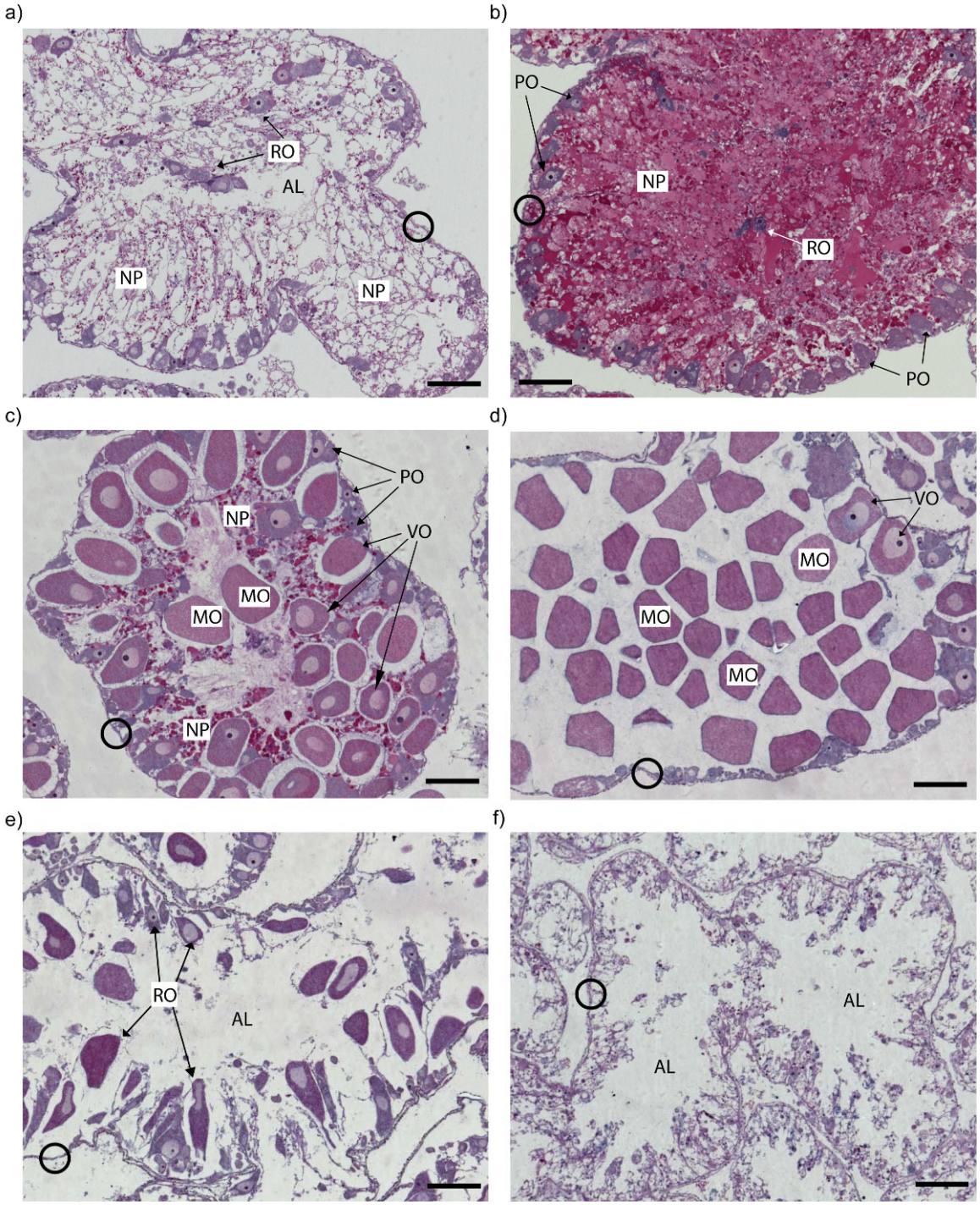

Figure 2: Ovarian sections of *Psammechinus miliaris* stained with Alcian Blue/PAS: a) stage I (recovery phase), NP start to form a meshwork across the acinus and new previtellogenic oocytes are present along the acinal wall. In this individual many relict oocytes in the resorbing process are present; b) stage II (growing phase), NP form a dense meshwork containing a high amount of PAS+ material; the previtellogenic oocytes present along acinal wall increase in size; c) stage III (premature phase), acini contain oocytes at all stages of development; vitellogenic oocytes detach from the acinal wall and move towards the lumen where mature ova accumulate; NPs still contain PAS+ granules but their content is decreased; d) stage IV (mature stage), mature oocytes accumulate in acinal lumen, nutritive phagocytes are reduced; e) stage V (partly spawned stage), of a premature animal with relict oocytes at different stages of development present in the acini; f) stage VI (spent stage), acini appear empty; some relict oocytes may be present in the resorbing process; no PAS+ material is present. Bar = 100 µm. Circle = acinal wall, AL = acinal lumen, MO = mature ova, NP = nutritive phagocytes, PO = previtellogenic oocytes, RO = relict oocytes, VO = vitellogenic oocytes.

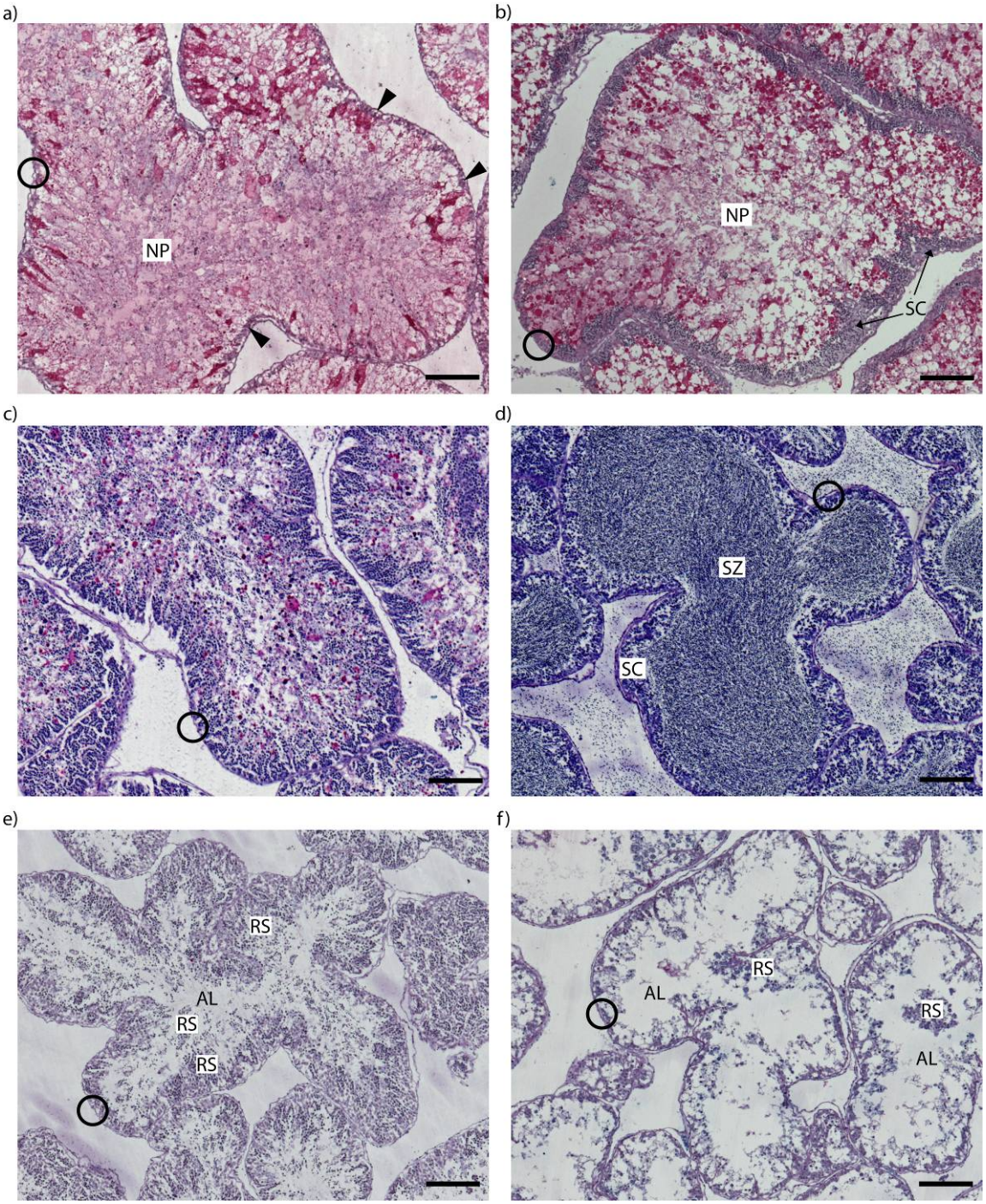

Figure 3: Testicular sections of *Psammechinus miliaris* stained with Alcian Blue/PAS. a) stage I (late recovery phase), NP form meshwork across acinus and accumulate PAS+ material; primary spermatocytes are present along the acinal wall (arrowheads); b) stage II (growing phase), the basophil layer increases in thickness and columns of spermatocytes project centrally towards acinal lumen, NPs contain a high amount of PAS+ material; c) stage III (premature stage), spermatozoa are present in the centre of the acini; NPs are reduced; d) stage IV (mature stage), acini are filled with mature spermatozoa and are largely devoid of NPs; e) stage V (partially spawned stage), spaces are vacated by spawned spermatozoa; residual sperm are present; f) stage VI (spent stage), testis are largely devoid of contents. Bar = 100 µm. Circle = acinal wall, AL = acinal lumen, NP = nutritive phagocytes, RS = residual sperm, SC = spermatocytes, SZ = spermatozoa.

Testicular stages

In males, acini contain a loose meshwork of nutritive phagocytes with vacuolated appearance during early recovery period (stage I). PAS+ material starts to accumulate in the nutritive phagocytes visible as magenta granules in Alcian Blue/PAS stain and purple (eosinophilic) granules in H&E. Testes may contain a high number of residual sperm in the resorbing process. With ongoing recovery of the tissue the NP meshwork becomes denser, PAS+ material increases and less relict sperm are found. At the end of the recovery period, spermatogonia are evident as narrow basophilic layer (10-20 µm) around the acinal wall (Figure 3a). The basophilic layer increases in thickness (10–70 µm) during the growing phase (stage II, Figure 3b). Columns of spermatocytes are present projecting towards the centre of the acini. The acini are filled with NPs containing intensely PAS+ and eosinophilic droplets. In the premature stage (stage III, Figure 3c), the spermatocyte columns increase in depth. Spermatids and spermatozoa are present in the acinal lumen displaying the NPs which are hardly discernible any longer. Spermatozoa can easily be identified by the triangular shape of their head and the presence of a tail. In mature (stage IV, Figure 3d) males, the acinal lumina are filled with mature spermatozoa and the NPs are limited to the periphery of the acini. Testes are largely devoid of eosinophilic material apart from a narrow band at the acinal wall. In animals which had partially spawned (stage V, Figure 3e), spaces vacated by shed sperm appear in the acini. Spent testes (stage VI, Figure 3f) are largely devoid of contents. A loose meshwork of NPs may be present at the periphery of the acini. Interestingly, female as well as male individuals being in the recovery period often contained high numbers of relict gametes in the resorbing process (Figure 2a).

As illustrated in Figure 2d most animals in the premature and mature stage had already spawned part of their gametes which is evident by empty spaces within the acini and spawned gametes in the gonoducts.

Seasonal variations of gonadal stages and abiotic factors

The relative frequencies of the gonadal maturation stages of *P. miliaris* are presented in Figure 4a. During the sampling period growing, premature and mature stages dominante from March till June. In 2007, premature animals were present from January till August whereas premature females were found from January till July and premature males from February till August. Only single mature individuals were found in May, June and August. In females, gametogenesis started in October which is evident by the occurence of individuals in the growing stage. In males, however, all individuals were still in the recovery period in October and November 2007. During

the growing period, primary oocytes started to grow in size in females while in males the basophilic layer of spermatogenic cells increased in thickness (Figure 2b and 3b).

During the sampling period, surface seawater temperature and daylength peaked in June with 17.9 °C and 17.3 hours, respectively (Figure 4b). When water temperature and day length decreased in the study area in October gametogenesis commenced.

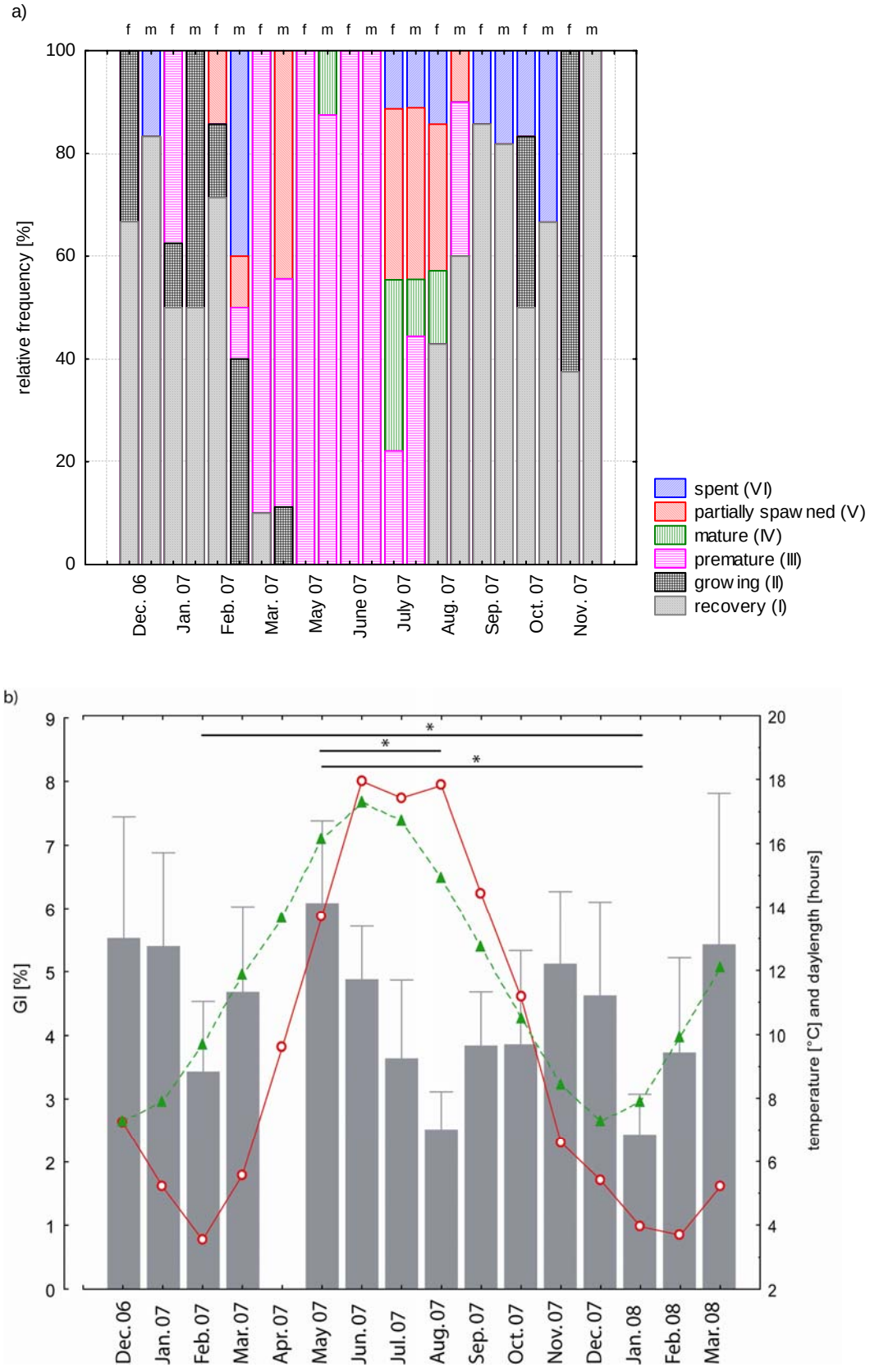

Figure 4: a) Relative frequencies of the reproductive stages of *P. miliaris* (f = females, m = males) from December 2006 to November 2007. b) Surface seawater temperature [°C] (red circles), day length [hours] (green triangles) as well as gonad indices (GI) of *P. miliaris* (mean ± standard deviations presented as grey bars) from December 2006 to November 2007 in the northern Wadden Sea. Asteriks indicate significant differences in the GI between months (Kruskal-Wallis ANOVA on ranks, $p < 0.001$, Dunn's test, $p < 0.05$).

Gonad indices

The gonad indices (GI) of *P. miliaris* showed no clear seasonal pattern at the sampling site (Figure 4a). The values varied from minimally 0.44 to maximally 16.15 % and peaked in May and November 2007 with a mean of 6.1 and 5.1%, respectively. In May 2007, the GI was significantly increased in comparison to August 2007 and January 2008 (Kruskal-Wallis ANOVA on ranks, $p < 0.001$, Dunn's test, $p < 0.05$). In January 2008, the GI was significantly reduced compared to January 2007 (Kruskal-Wallis ANOVA on ranks, $p < 0.001$, Dunn's test, $p < 0.05$). No significant difference was found in the GI of female and male sea urchins (t-test, $p = 0.079$).

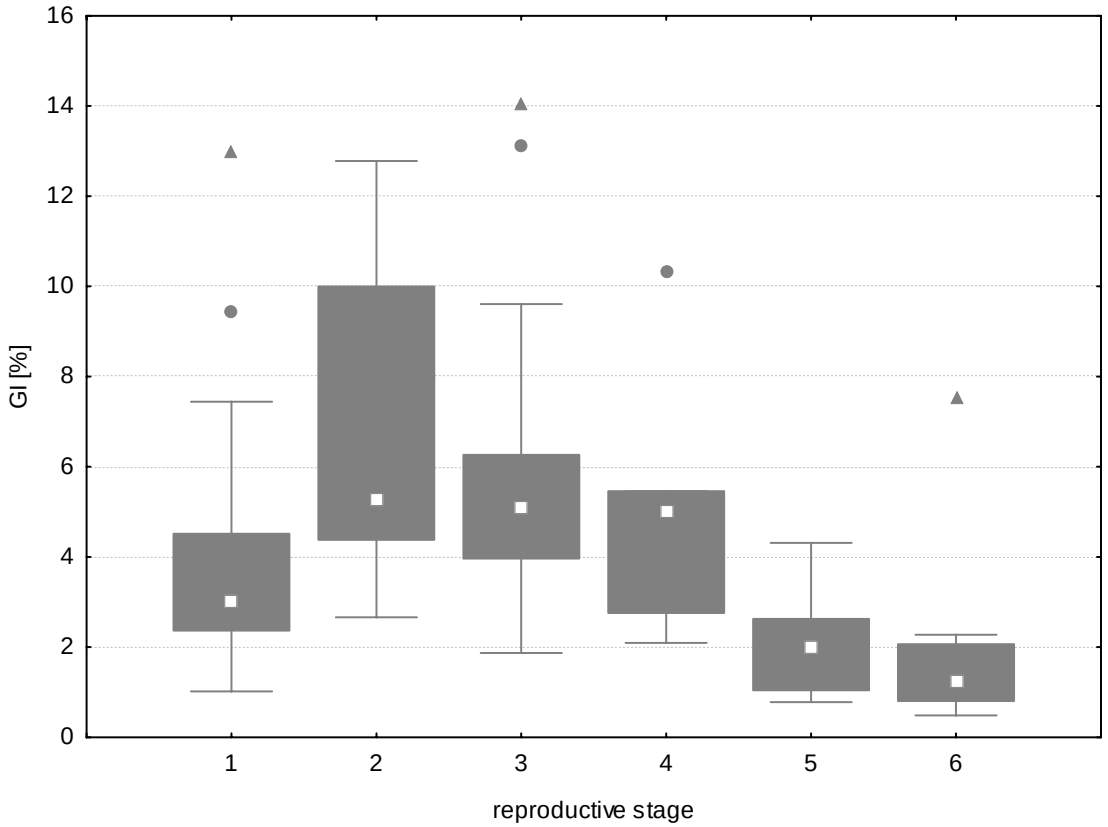

Figure 5: Gonad indices (GI) [%] of *Psammechinus miliaris* in dependence of the different reproductive stages. Data are presented as box-whisker plot with median values and the 25% and 75% percentile. Dots indicate outliers and triangles extreme values. 1 = recovery, 2 = growing, 3 = premature, 4 = mature, 5 = partially spawned, 6 = spent. Different letters indicate significant differences between reproductive stages (one-way ANOVA, $p < 0.001$, Tukey HSD, $p < 0.05$).

However, the reproductive stage had a significant effect on the GI of *P. miliaris.* (Figure 5, one-way ANOVA, reproductive stage, $p < 0.001$, Tukey HSD, $p < 0.05$). Animals in the growing and premature stage had significantly increased GI compared to animals in the recovery, partially

spawned, or spent stage. Mature animals had higher GI values than partially spawned and spent animals.

Lipofuscin deposition

As already described by Schäfer and Köhler (2009a), lipofuscin was identified as irregularly shaped and heterogeneously textured blue granules in sections stained with the Schmorl's technique. Lipofuscin was primarily localized in extracellular spaces in the acini and inside of NPs. In addition, lipofuscin was identified in epithelial cells of the acinal wall and outside of the acini close to cellular debris. No lipofuscin was observed in gametogenic cells. In male *P. miliaris,* however, lipofuscin was often formed at sites were spermatocytes had undergone necrosis (compare arrows in Figure 8e).

Lipofuscin content in the gonads of *P. miliaris* varied significantly depending on the reproductive stage and the sex of the animals (Figure 6, 2-way ANOVA, without interaction, sex: $p < 0.001$, reproductive stage: $p < 0.001$). Lipofuscin accumulation increased in both sexes from lowest values during the growing period to highest values in the spent stage. Individuals in the recovery phase had lower lipofuscin content than the ones in the spent stage. Males showed significantly increased lipofuscin content in comparison to females. No interactive effect was found between reproductive stage and sex (2-way ANOVA, $p = 0.8428$) indicating that the underlying mechanisms leading to lipofuscin deposition in the gonads of *P. miliaris* are the same in both sexes. The size of the animals, measured as vertical test diameter, did not correlate with lipofuscin content (Spearman Rank Order Correlation, $p = 0.111$, $N = 174$).

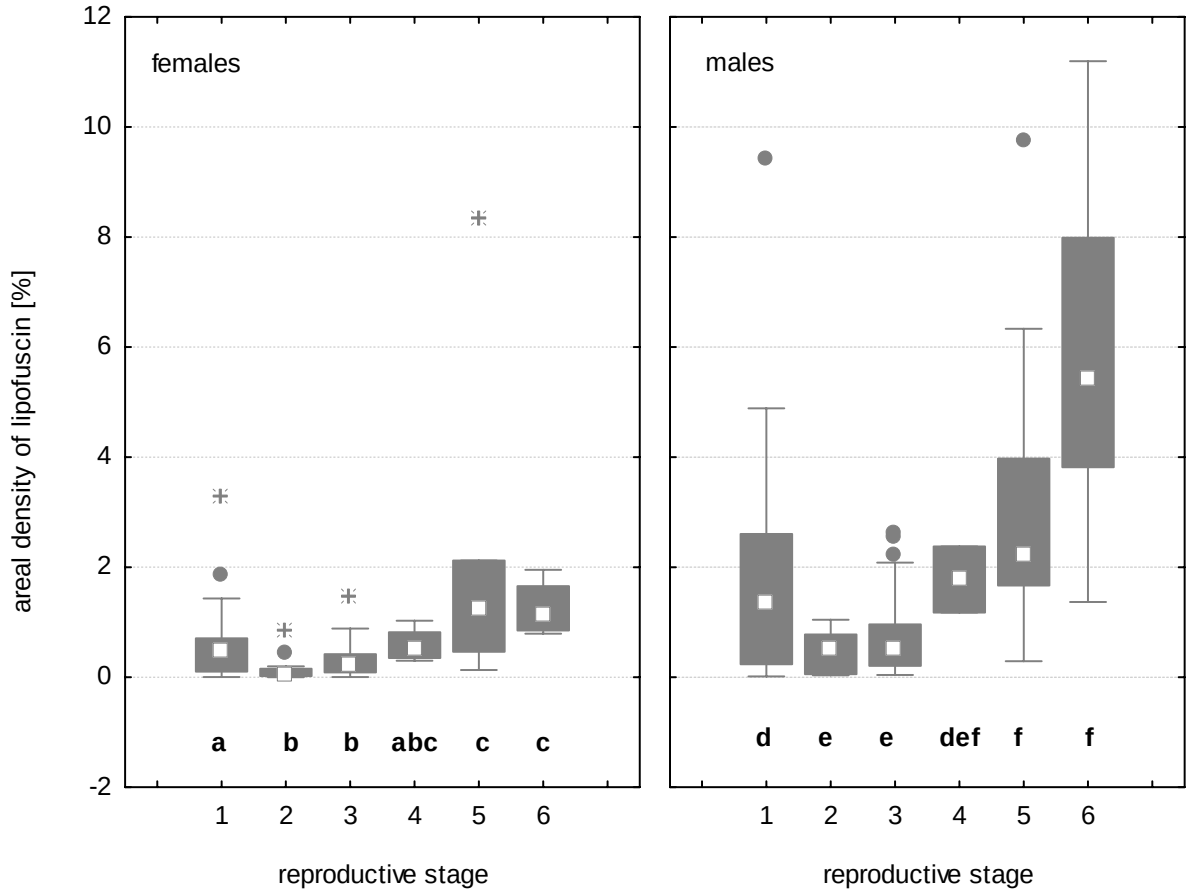

Figure 6: Lipofuscin content measured as areal density [%] in gonadal sections of *P. miliaris* in the different reproductive stages. Data are presented as box-whisker plots with median values and the 25% and 75% percentile. Dots indicate outliers and asteriks extreme values. Left box: females, right box: males. Reproductive stages: 1 = recovery, 2 = growing, 3 = premature, 4 = mature, 5 = partially spawned, 6 = spent. Different letters indicate significant differences between reproductive stages (2-way ANOVA, $p < 0.001$). Lipofuscin content in males is significantly higher than in females ($p < 0.001$). There is a significant difference between the reproductive stage ($p < 0.001$) but there is no interactive effect between reproductive stage and sex ($p = 0.8428$).

PAS+ material

PAS+ content was significantly different depending on the reproductive stage and the sex of the animals (2-way ANOVA, sex: $p = 0.0025$, reproductive stage: $p < 0.001$, interaction: $p = 0.0007$, Figure 7). Females incorporated significantly more PAS+ material than males. Additionally, PAS+ content was highest in the recovery and growing stages, in both sexes. In males, PAS+ content was very low during the rest of the reproductive cycle. In females, however, medium values were found in mature animals which can be addressed to vitellogenic oocytes containing many small, intensely PAS+ stained granules which were identified as yolk (e.g. Figure 1c). Despite their small size, the yolk granules were detected by the image analysis system at 100x magnification.

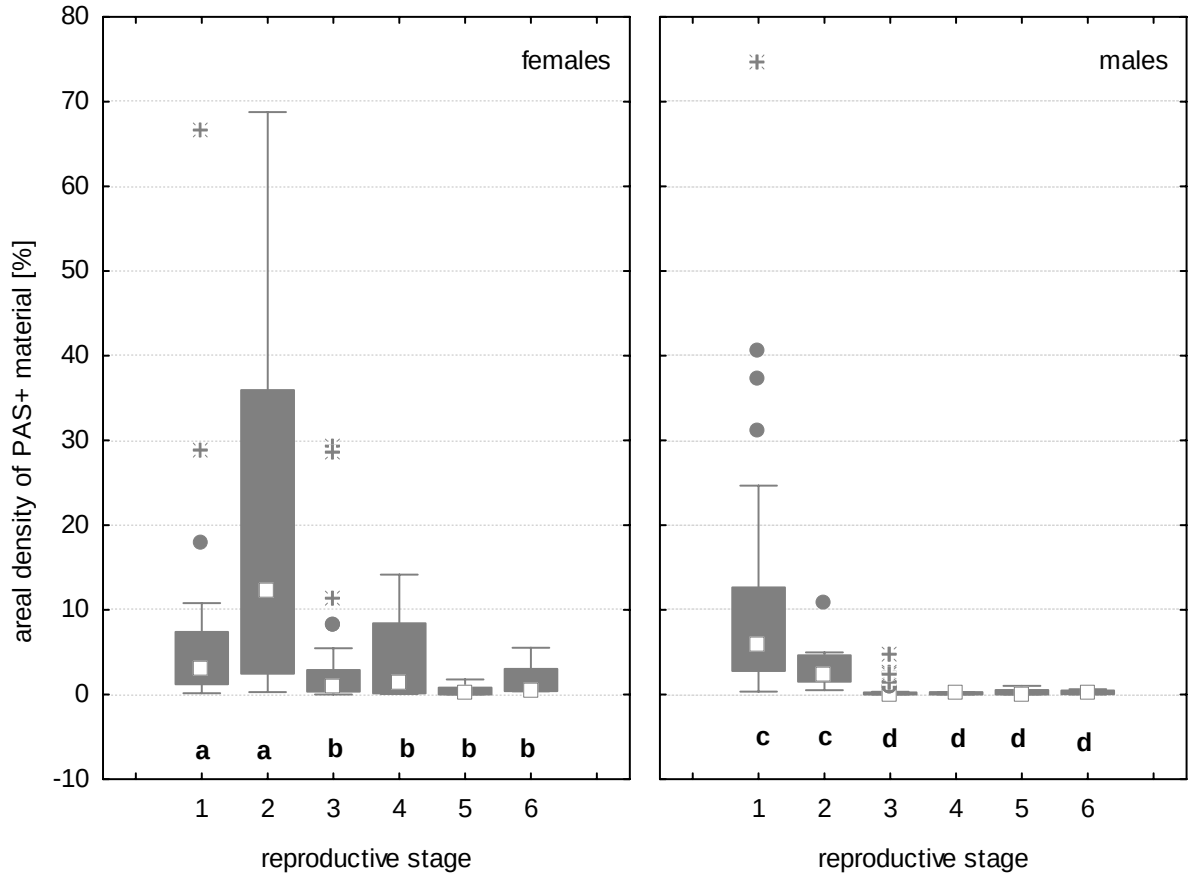

Figure 7: Content of PAS+ material measured as areal density [%] in gonadal sections of *P. miliaris* in the different reproductive stages. Data are presented as box-whisker plots with median values and the 25% and 75% percentile. Dots indicate outliers and asterisks extreme values. Left box: females. Right box: males. Reproductive stages: 1 = recovery, 2 = growing, 3 = premature, 4 = mature, 5 = partially spawned, 6 = spent. Different letters indicate significant differences between reproductive stages (2-way ANOVA, $p < 0.001$). PAS+ content in males is significantly lower than in females ($p = 0.0025$). Reproductive stage has a significant effect on PAS+ levels in *P. miliaris* ($p < 0.001$). There is also an interactive effect of sex and reproductive stage ($p<0.001$).

Gonadal anomalies and pathologies

The acinal wall is composed of two layers of epithelial cells which can be recognized by their basophilic nuclei. Depending on the plane of tissue sectioning, at least one further eosinophilic layer is visible which may be regarded as collagen (Schäfer and Köhler 2009a). In some animals the epithelial layers were often apart from each other. This dilation was frequently accompanied with the augmentation of collagenous material between the epithelial layers, so called fibrosis (Figure 8a). Concomitantly, with fibrosis of the acinal wall hyperplasia and hypertrophy of the epithelial cells was often found (Figure 8b). In partially spawned or spent animals, fibrosis of the acinal wall was significantly more frequent than in the other reproductive stages (Kruskal-Wallis one way ANOVA, multiple comparison, $p < 0.0001$).

Furthermore, we observed degenerative processes of germ cells. In females, oocytes undergoing atresia are characterized by fading and vacuolisation of the cytoplasm, lysis of the nuclear membrane and finally cell lysis as previously described in detail elsewhere (Schäfer and Köhler 2009a). In males (Figure 8e), degenerating sperm showed shrinking and darkening as well as fragmentation of the nucleus. Oocyte atresia and sperm necrosis were significantly increased in partially spawned compared to premature animals (tested were data from premature, mature and partially spawned animals; oocyte atresia: Kruskal-Wallis ANOVA on ranks, $p = 0.0190$, multiple comparison procedure; sperm necrosis: Kruskal-Wallis ANOVA on ranks, $p < 0.0001$, multiple comparison procedure). In premature females, oocyte atresia was elevated but not significantly different in individuals which had undergone untimely spawning compared to individuals which had not released any gametes (Kruskal-Wallis ANOVA on ranks, $p = 0.0559$). In premature males, spawning had no effect on sperm necrosis (Kruskal-Wallis ANOVA, $p = 0.647$).

In premature males, vacuolisation of the NPs was observed with a high prevalence (74%, N = 27, Figure 8d, e). Furthermore, in some males in the growing or the premature phase the basophil layer detached from the acinal wall (Figure 8f). This phenomenon was prevalent in 38% of the males in the growing stage (N = 8) and in 29% of the males in the premature stage (N = 28). Single sites of deposited proteinaceous material, stained magenta with Alcian Blue/PAS, were found between epithelial layers of the acinal wall as well as inside or outside of acini (17%, N = 174, not presented). Both sexes also exhibited disorganisation of the acinal structure which was evident by early maturational stages (spermatogonia/oogonia and primary spermatocytes/oocytes) present in the centre of the acini (Figure 8c). Disorganisation of the acinal structure was a relatively common finding which was prevalent in around 20% of the animals being in the growing or the premature stage (growing phase: n = 4, N = 20; premature: n = 12, N = 60).

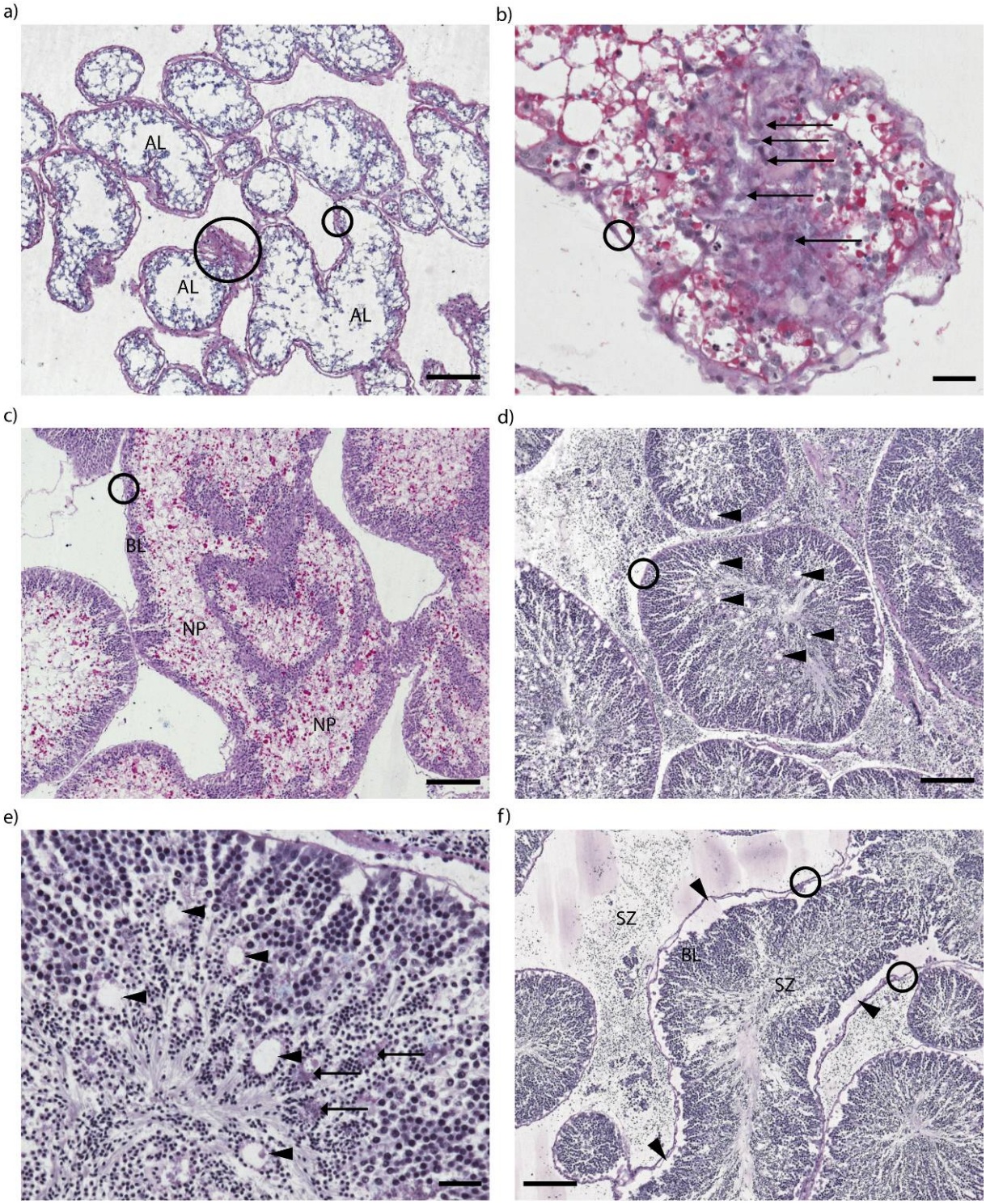

Figure 8: Testicular sections of *Psammechinus miliaris* stained with Alcian Blue/PAS showing tissue anomalia. a) Indivual in the spent stage with severe fibrosis of the acinal wall (circles); b) detail of fibrosis of the acinal wall showing hyperthrophy and hyperplasia of the epithelial cells (arrows); c) acinus of one individual in the growing stage with basophilic cells in the acinal lumen; d) individual in the premature stage with severe vacuolisation of the NPs (arrowheads); e) detail of figure d) with vacuolisation of NPs (arrowheads) and sperm necrosis (arrows); f) premature individual with severe dilation of the basophil layer from the acinal wall. Bars: a), c), d) and f) 100 µm; b) and e) 20 µm; circles = acinal wall. AL = acinal lumen, BL = basophil layer, NP = nutritive phagocytes, SZ = spermatozoa.

Discussion

The present study demonstrates the importance for critical and careful analysis and interpretation of histological and histochemical findings in reproductive tissues. In sea urchins we identified gonadal stage, sex as well as spawning as potential confounders of laboratory or field studies investigating xenobiotically mediated effects on gonads.

Lipofuscin is widely used as biomarker for oxidative stress induced by exposure to various environmental pollutants in fish (liver) and mussels (digestive gland) (reviewed in Au 2004; Viarengo et al. 2007). In the reproductive tissue of the sea urchin *Strongylocentrotus intermedius* inhabiting polluted areas in the Sea of Japan lipofuscin accumulation has previously been related to heavy metal contamination (Vashchenko and Zhadan 1993; Vaschenko et al. 2001; Vashchenko et al. 2001). Though, in laboratory studies with *P. miliaris* we did not find increased deposition of lipofuscin in the ovary after exposure to the polycyclic aromatic hydrocarbon phenanthrene (Schäfer and Köhler 2009a) or the heavy metal lead (Schäfer and Köhler 2009b). The present study, in fact, shows that the lipofuscin content in gonads of sea urchins is significantly dependent on the reproductive stage of the animals with highest levels found in the partially spawned and spent individuals. Similarly, in fish ovaries lipofuscin/ceroid is formed in conjunction with degeneration of follicular cells after completion of vitellogenesis (Blazer 2002). Further, Miranda et al. (1999) observed lipofuscin accumulation in the final stage of follicular atresia in female teleosts. It is likely that lipofuscin being formed and accumulated during gametogenesis is released from the gonads. Otherwise, larger animals which had already undergone gametogenesis in previous years would have higher lipofuscin content than their smaller and younger conspecifics. Since levels of lipofuscin decrease after spawning we suppose that lipofuscin is released during the recovery period.

Furthermore, male *P. miliaris* exhibited significantly higher levels of lipofuscin than females. In another study, we found significantly lower concentrations of ascorbate in testes of *P. miliaris* compared to ovaries (Schäfer et al. unpublished data). In general, testes of different animal phyla are known to contain a high amount of polyunsaturated fatty acids which are prone to oxidation (Halliwell and Gutteridge 2007). In *P. miliaris* significantly increased levels of polyunsaturated acids were found as well as a dramatic reduction in the fatty acids 22:6(n-3) and 22:5(n-3) with increasing maturity stage (Hughes et al. 2006). Ovaries may, therefore, be less susceptible to oxidative stress than testes due to a higher antioxidant scavenging potential and the presence of

less oxidizable compounds. This may result in less lipid and protein peroxidation and finally lipofuscin deposition in ovaries than testis.

The high PAS+ content in the gonads of *P. miliaris* during recovery and growing period and their decrease during gametogenesis reflect the storage of nutrients in NPs and their consumption or packaging in developing oocytes (Brooks and Wessel 2002; Unuma et al. 2003; Walker et al. 2005). The higher levels of PAS+ material found in female *P. miliaris* can be explained by vitellogenesis of oocytes which accumulate PAS+ yolk with ongoing maturation. Spermatogonia and subsequent stages of spermatogenesis are, however, PAS negative (compare Figure 3 b-d and Walker et al. 2005). Furthermore, the MYP content, the major PAS+ compound in sea urchin gonads, decreases in both sexes during gametogenesis with lowest levels found in mature males (Unuma et al. 2003). The proteinaceous material observed in individual sea urchins may be analog to deposition of proteinaceous fluids in fish where they have been shown to be associated with exposure to endocrine disruptive compounds (Wolf 2005).

In the present study, we identified fibrosis of the acinal wall which occured significantly more often in partially spawned or spent animals than in the other gonadal stages. The thickness of the gonadal wall of sea urchins has previously been reported to change during the year (Palmer Wilson 1940). Gonadal fibrosis may further be induced in sea urchins (Schäfer & Köhler 2009) and fish (Blazer 2002; Kang et al. 2002; Palace et al. 2002; Dietrich et al. 2009) after exposure to environmental pollutants. Since degeneration and phagocytosis of relict germ cells is very prominent in partially spawned or spent sea urchins and the tissue is reorganised after spawning, fibrosis may be regarded as regenerative repair in sea urchin gonads as suggested by Dietrich et al. (2009) for reproductive tissue of fish.

As mentioned, we found degeneration of germ cells preferably in the later developmental stages of *P. miliaris* which is similar to studies with fish (Dietrich et al. 2009). At the end of the breeding season follicular atresia in fish is considered as a “clean-up” process (Dietrich et al. 2009), whereby the somatic follicle cells phagocytose the yolk as well as mitochondria and other organelles of the oocytes (Miranda et al. 1999). Similarly, in sea urchins phagocytosis of relict sperm and shrinkage of nutritive phagocytes has been described in postspawned testes (Reunov et al. 2004). However, the mechanisms initiating and regulating degeneration of germ cells in aquatic organisms are still poorly understood. In atretic oocytes of teleosts, Miranda et al. (1999) found signs of necrosis such as dissolution and disappearance of the nucleus and changes in organelles. Though, other studies

also discuss the involvement of apoptosis in degeneration of germ cells (Sakamaki 2003; Hussein 2005). In male *P. miliaris* we found degenerating sperm characterized by nuclear changes such as pyknosis (shrinking and darkening) and karyorrhexis (fragmentation of the nucleus) comparable to findings described as necrotic sperm in fish (Blazer 2002). Though, Baltus et al. (2006) assigned spermatocytes in testes of mice, exhibiting the same features like the sperm in our study, as apoptotic.

The vacuolisations observed in 74% of premature males are phagocytotic processes of sperm which have previously been described by Reunov et al. (2004). In both sexes of sea urchins degeneration and phagocytosis of germ cells at the end of the seasonal cycle may be regarded as a recycling process to meet the requirements for basal metabolism. It needs to be further evaluated whether necrosis or apoptosis are the underlying mechanisms of germ cell degeneration in sea urchins.

For histopathological assessment of toxicologically induced changes in the gonads it has to be considered that suitable gametes are only present in the growing, premature and mature stages (Schäfer and Köhler unpublished). The present study shows that *P. miliaris* from the northern Wadden Sea can be used for histological analyses of the gonads in the scope of experiments or biomonitoring studies from March till June. Furthermore, it confirms that *P. miliaris* has an annual reproductive cycle with one spawning period during the summer months as already described by Kelly (2000). Though, the timing of the different reproductive stages differ in *P. miliaris* from the northern Wadden Sea and at the west coast of Scotland: Kelly (2000) found *P. miliaris* in the growing stage not before March, whereas we recorded first individuals in the growing stage in October already. Furthermore, in Scotland premature animals were only found in May/June whilst they were present from January till August in the Wadden Sea. In a laboratory study with one-year old hatchery-reared *P. miliaris,* Kelly (2001) showed that lengthening days are an important cue for completion of gametogenesis. In detail, significantly more individuals reached maturity after treatment with increasing photoperiod mimicking natural advancing spring day lengths at the relevant latitude. Furthermore, fewer females reached maturity after treatment with fixed temperatures of ≥ 9°C indicating that temperatures below 9°C during winter may be required for completion of vitellogenesis (Kelly 2001). Increasing day length and temperature can, however, not be considered to be the triggers for commencing gametogenesis for the population in the northern Wadden Sea.

Notably, we found many relict gametes in the resorbing process in *P. miliaris* during recovery period compared with other studies of sea urchins (compare micrographs in e.g. Byrne 1990; Spirlet et al. 1998; Kelly 2000) indicating that spawning may have been incomplete during summer. Recycling of a large amount of nutrients from many relict gametes may have promoted gametogenesis in *P. miliaris* in the northern Wadden Sea. Furthermore, increased water temperatures due to climate change may influence timing and commencing of gametogenesis as well as spawning. Based on data from 1984 to 2005, Martens and van Beusekom (2008) calculated that monthly water temperature increased by 0.04 °C in the List Tidal Basin which was mainly attributed to an extension of the warm summer period till September. Additionally, the years 2007 and 2008 showed the highest mean annual temperature since 1949 (van Beusekom JEE, Wadden Sea Station Sylt (Germany), pers. communication). Further studies are needed to clarify if increasing sea water temperatures affect the reproductive cycle as well as spawning events in sea urchins.

Nonetheless, population-specific differences in the regulation of gametogenesis may be responsible for the observed differences between the Scottish and the German population of *P. miliaris*: At the west coast of Ireland, Byrne (1990) found that subtidal *Paracentrotus lividus* exhibited a longer period of reproductive maturity possibly due to a better nutritional status compared with an intertidal population. However, Kelly (2000) found no differences in the reproductive cycle of *P. miliaris* at two replicate sites in littoral and subtidal habitats.

In the present study, the gonad indices show not a clear seasonal cycle confirming that GI alone cannot be used for determining the reproductive stage of sea urchins (e.g. Lozano et al. 1995). However, the gonad indices of *P. miliaris* significantly correlate with the reproductive stage of the animals with highest values in growing and premature animals. Gonad indices decrease as spawning events occur resulting in lowest values in spent animals. Possibly, different reproductive stages present in specific months superimpose seasonal trends in the GI of *P. miliaris* in the present study.

For the evaluation of histological or histochemical changes in toxicological experiments and biomonitoring studies detailed knowledge about the natural variability of the tissue is required. In gonads of sea urchins we identified gonadal stage, sex and spawning as potential confounders. Therefore, we recommend to critically and carefully interpret alterations in sea urchin gonads only after inclusion of these factors.

Acknowledgements

This study was financed by the Alfred Wegener Institute for Polar and Marine Research within the Helmholtz Association. We thank Birgit Hussel and the crew of the MS Mya on the Island of Sylt for sampling of the sea urchins. We are grateful to Justus van Beusekom for kindly providing the seawater temperature data and the figure with the sampling sites. Moreover, we thank Tom Brey for his help with the statistical analysis of the data and Christiane Schäfer from the Zeiss Company for her help with the Image Analysis. We also acknowledge the work of two students, especially of Yvonne Sakka, who prepared and analysed part of the samples.

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Gonadal lesions of female sea urchin (*Psammechinus miliaris)* after exposure to the polycyclic aromatic hydrocarbon phenanthrene

Schäfer, S., Köhler, A., *published in* Marine Environmental Research (2009) 68: 128-136.

Abstract

Histopathological alterations in the reproductive tissue of the female sea urchin *Psammechinus miliaris* after exposure to the polycyclic aromatic hydrocarbon (PAH) phenanthrene were investigated using light microscopy. Sexually mature sea urchins were exposed to 500 µg L^{-1} phenanthrene for 20 days. A severe disorganisation of the acinal structure of the gonads was observed in phenanthrene-treated animals, mainly as a result of aggregates of degenerating previtellogenic oocytes. Growth and maturation of previtellogenic oocytes were inhibited, whereas there were no obvious effects on vitellogenic oocytes and mature ova. The acinal wall showed signs of fibrosis as well as dilation of the two epithelial layers. Phenanthrene severely affected the reproductive function of the sea urchins since a proper oogenesis of the ovarian tissue is very unlikely. Generally, the observed alterations are similar to histopathological changes in reproductive tissue in other invertebrates after exposure to PAHs and oil.

Key words

histopathology, gonad, sea urchin, oocyte atresia, phenanthrene, *Psammechinus miliaris*, lipofuscin

Introduction

Many environmental pollutants have been shown to interfere with reproductive function in animals (Haschek and Rousseaux 1998). Sea urchin gametes and embryos are widely used for evaluating biological effects of contaminants in marine environments (Greenwood 1980; Kobayashi 1980; Hose 1985; Klöckner et al. 1985; USEPA 1993). However, the underlying mechanisms resulting in reduced reproductive success of adult marine invertebrates and of sea urchins, in particular, are hardly investigated (Au et al. 2001a; Au et al. 2001b). Histopathology is

a well established tool for the identification of tissue and cellular injury. Histopathological biomarkers and cellular changes in the reproductive tissue can be used to gain a better understanding of the adverse effects of environmental pollutants on reproduction (Au 2004; Dietrich et al. 2009).

Sea urchins play a key role in benthic communities of coastal waters and changes in sea urchin populations have led to major alterations in marine community structure (Scheibling 1986; Steneck et al. 2002). *Psammechinus miliaris* is a common echinoid found at depths of up to 100 metres in the Atlantic from Scandinavia to the Azores including the North Sea and the Baltic Sea (Boschma et al. 1932; Campbell 1977). This species has been used as a test organism in fertilisation and embryo development assays (Klöckner et al. 1985; Caldwell et al. 2002; Caldwell et al. 2004). In the past years, *P. miliaris* has gained interest as promising candidate for aquaculture especially in polyculture with the Atlantic salmon (Kelly et al. 1998).

Sea urchins are dioecious and due to their pentaradial body organisation they possess five gonads with separate gonadopores (Westheide and Rieger 1996). Functional units of the gonads are the so-called acini which are surrounded by a multi-layered acinal wall (Palmer Wilson 1940). Germ cells are formed at the germinal epithelium of the acinal wall and migrate towards the lumen of the acinus during maturation (e.g. Byrne 1990; Walker et al. 1998; Kelly 2000). During oogenesis, oocytes undergo meiotic divisions and an increase in size to form mature ova. In the ovary, oogenic cells, namely oogonia, previtellogenic and vitellogenic oocytes, can be distinguished from mature ova by the presence of the germinal vesicle (GV) containing a prominent nucleolus (Chatlynne 1969). Furthermore, nutrients are supposed to be transferred from the somatic cells of the gonads, the nutritive phagocytes (NPs), to the gametogenic cells (Walker et al. 1998). Therefore, vitellogenic oocytes and mature ova have - in contrast to the early maturational stages, the oogonia and previtellogenic oocytes - yolk granules in their cytoplasm. In most sea urchin species living at higher latitudes, gametogenesis is a single annual event with one spawning period (Walker et al. 1998). At the west coast of Scotland *P. miliaris* is reported to have a clearly defined reproductive cycle with a single spawning period in June and July (Kelly 2000).

There are only few studies investigating the effects of chemicals on gonadal tissue of sea urchins. At the light microscopical level histopathological lesions of sea urchin gonads are reported by Vashchenko and Zhadan (1993), Vaschenko et al. (2001) and Vashchenko et al. (2001) in areas of Peter the Great Bay (Sea of Japan) polluted with heavy metals. Histopathological alterations of

spermatogenesis in the sea urchin *Anthocidaris crassipina* after experimental exposure to phenol and cadmium were investigated using electron microscopy (Au et al. 2001b; Au et al. 2003).

Polycyclic aromatic hydrocarbons (PAH) are lipophilic organic compounds which are ubiquitously found in the marine environment. Phenanthrene is a low molecular weight, 3-ring PAH and classified as a priority pollutant by the United States Environmental Protection Agency (US EPA). It is an important petroleum-source PAH and a major component of the total content of PAH compounds in the marine environment. PAHs are known to induce degeneration of oocytes and ovarian lesions in several species (*Mytilus edulis* - Aarab et al. 2004; *Littorina littorea* - Cajaraville et al. 1990; mammals - Sakamaki 2003). Degeneration and absorption of oocytes, so called oocyte atresia, is a normal physiologic event which may become pathologic after chemical exposure (Blazer 2002; Dietrich et al. 2009). Furthermore, contamination with PAHs has been shown to be associated with an increased accumulation of lipofuscin in marine organisms (Krishnakumar et al. 1994; Krishnakumar et al. 1997; Au et al. 1999; Au 2004). Lipofuscin, also known as age pigment, is widely regarded as end product of protein and lipid peroxidation due to oxidative stress (Au et al. 1999; Au 2004; Terman and Brunk 2004). In the sea urchin *Lythechinus variegatus* phenanthrene significantly retarded embryo development at environmentally relevant concentrations (Steevens et al. 1999). In *Lythechinus anemensis*, phenanthrene disrupted axial development in embryos in a dose-dependent fashion which could be linked to nuclear accumulation of the protein β-catenin (Pillai et al. 2003). Pillai et al. (2003) have shown that among five investigated PAHs phenanthrene was most potent in causing exogastrulation in sea urchin embryos.

The aim of the present study was to give a detailed description of the histopathological alterations of the reproductive tissue of the female sea urchin (*P. miliaris*) after exposure to phenanthrene. The findings are compared with effects of hydrocarbons on the reproductive tissue of other aquatic invertebrates. Changes of the general acinal structure, effects on the different developmental stages of oocytes and the nutritive phagocytes, as well as alterations of the acinal wall are described. Histopathological alterations of the gonads such as aggregates of degenerating oocytes, the percentage of degenerating oocytes, lipofuscin accumulation and dilation of the acinal wall are quantitatively or semi quantitatively assessed. Furthermore, the gonad weight and the gonadosomatic index in the different treatments are compared.

Materials & Methods

Animal collection and experimental set up

Adult *P. miliaris* were collected with a beam trawl close to the Island of Sylt (Germany) at 55° 02, 40N and 08° 27, 25E in May 2006. They were separated by sex and transported to the laboratory in Bremerhaven. In a range finding experiment, sea urchins were exposed to set concentrations of 1, 3, 12, 42, 145 and 500 µg L^{-1} phenanthrene, to 0.01% acetone, as the solvent control, and to sea water only for 10 days. Each treatment consisted of two replicate tanks (glass vessels with a volume of 1.5 L) with 4 sea urchins each. Since no mortality occurred in this preliminary experiment 500 µg L^{-1} phenanthrene was chosen as sublethal test concentration for the subsequent experiment. Female sea urchins of a minimum size of 20 mm horizontal test diameter were selected and acclimatized to experimental conditions for 4 weeks. Sea urchins were exposed to 500 µg L^{-1} phenanthrene (dissolved in acetone), to 0.01% acetone, as solvent control, and to sea water only (9 ± 0.5 °C, 33 ± 1 PSU, continuous aeration). Duration of the experiment was limited to 20 days to terminate the test before the natural spawning period of the animals had started. Each treatment consisted of 3 replicate tanks (16 L glass aquaria) containing 10 sea urchins each. Water was renewed every third day and phenanthrene or solvent were added at the respective concentrations. Sea urchins were fed with fresh spinach. Mortality of sea urchins was checked daily.

Sampling

For comparison of growth parameters, 24 samples were taken at the beginning (day 0) and 30 replicate samples per treatment were taken at the end of the exposure period (day 20). Animals were weighed and horizontal and vertical test diameters were measured with callipers. The animals were dissected and the gonads were removed and weighed. The gonad index (GI) was calculated as the wet weight of the gonad divided by the total somatic wet weight of the sea urchin expressed as percentage.

For histopathology, six replicate samples were taken per treatment at day 20. Gonads were immediately fixed in Baker's Formol calcium (4% formaldehyde and 2% calcium acetate) over night. On the next day, the samples were transferred into gum sucrose (30% sucrose and 1% gum arabicum) and stored at 4 °C.

Embedding, sectioning and staining

Gonad samples were dehydrated with an increasing series of acetone of 70% and 100% and embedded in methacrylate as described elsewhere (Köhler 2004). After the embedding procedure, tissue blocks were left to dry for at least two days and serial sections of 2 µm thickness were cut on a microtome (HM Leica RM 2145).

The sections were stained with hematoxylin and eosin (H&E) for demonstration of eosinophilic and basophilic structures, with Alcian Blue/PAS for neutral and acidic mucopolysaccharides (glycosaminoglycans) such as hyaluronic acid, chondroitin sulfate and sialic acids, with Schmorl's staining procedure for lipofuscin, and with Picrosirius for collagen. Most H&E staining procedures proved to be insufficient possibly due to the low eosinophilia of the tissue. The optimal method was the one described by Cerri and Cerri-Sassi (2003) which is appropriate for methacrylate embedded tissue.

For the Alcian Blue/PAS staining procedure, sections were first stained with Alcian Blue according to Lendrum et al. (1972). Then the slides were placed overnight in aldehyde blocking solution (2% sodium chlorite in 6% acetic acid). They were washed in running tap water for 10 min, rinsed in distilled water for 2 min, placed in 1% periodic acid for 10 min and rinsed again in distilled water for 5 min. Then the sections were counterstained in Schiff's reagent for 20 min, bleached in sulfurous acid for 2 min, washed again in distilled water for 5 min and stained with Gill's hematoxylin for 15 min. The sections were blued in running tap water for 10 min, rinsed in distilled water for 2 min, air dried and mounted with Euparal. The procedure results in blue staining of acidic mucopolysaccharides, magenta staining of neutral mucopolysaccharides and in a blue/purple staining of a mixture of acidic and neutral mucopolysaccharides. Nuclei appear deep blue.

For the Schmorl's procedure, sections were stained according to Pearse (1985) with minor modifications: sections were brought to water, stained in staining solution (freshly prepared 0.5% ferric chloride and 0.5 % potassium ferricyanide in distilled water) for 15 min, washed in 1% acetic acid for 2 min, washed in running tap water for 10 min and rinsed again in distilled water. Slides were left to dry and mounted in Euparal. A staining time of 15 min proved to be optimal and resulted in blue staining of lipofuscin granules. Longer staining resulted in background staining and staining of lipochromes present in yolk platelets.

For the identification of collagen fibres sections were stained with Picrosirius according to the method described by Cerri and Cerri-Sassi (2003) which resulted in homogeneous pink staining of collagen.

Microscopy

Slides were viewed with a microscope (Axioscope, ZEISS) and images were taken with a camera (MRc, ZEISS) coupled to a computer equipped with the software AxioVision (Version 4.6.3.0, ZEISS).

Tissue sections stained with the Schmorl's method were quantitatively and objectively assessed for lipofuscin content using computer assisted image analysis. The image analysis consisted of the above described microscope and camera and the software KS300 (Version 3.0, ZEISS). Image intensity was measured using grey values from 1 (clear, white) to 255 (dark, black). Three black and white images were randomly taken from each duplicate section of gonadal tissue (6 measurements per individual) with a 10x objective. In a special macro individual ovarian acini were marked as 'region of interest'. Lipofuscin granules within the defined acini were identified with the aid of a threshold function which discriminates the darker stained lipofuscin granules from the background. The software computed the areal density of lipofuscin [%] within the marked acinus ('region of interest') and mean values for each individual were calculated.

Further quantifications were performed on sections stained with Alcian Blue/PAS method. Aggregates of degenerating oocytes were quantified by applying an eyepiece graticule with a 100 squares grid (10 x 10) as an overlay. The number of squares lying on the acinus and the number of squares lying on aggregates of degenerating oocytes were counted at 400x magnification. In each duplicate section, five areas were randomly chosen (10 measurements per animal). The areal density of oocyte aggregates [%] within the acini was calculated. Dilation of the acinal wall was semi quantitatively assessed. For this purpose, in each sample 5 randomly chosen areas were graded according to a scaling scheme from 0 (no change) to 2 (severe alteration) at 100 x magnification. Degenerating previtellogenic oocytes identified by fading and vacuolisation of the cytoplasm, cell lysis and disruption of the membrane of the germinal vesicle at 600 x magnification were counted. Randomly selected previtellogenic oocytes of 100 were counted per sample and the data are presented as percentage. Oogonia were not considered due to their small size and the resultant difficulty in diagnosis.

Statistics

Residuals were tested for normality and variance homogeneity (Levene's test). Data on gonad weight and gonad index were box cox-transformed and a one-way ANOVA with Tukey HSD as post-hoc test were run. The data for aggregates of degenerating oocytes and lipofuscin content were square-root transformed. For the data on histopathology, a one-way ANOVA was run with the Holm-Sidak method as post-hoc test. In case of non-normal data, a Kruskal-Wallis ANOVA on ranks with a Dunn's test as post-hoc test was used. The significance level was set at $p < 0.05$. For the growth data tests were run with JMP (Version 7.0, SAS Institute Inc.) while histopathological analysis was done with Sigma Stat (Version 3.0.1, SPSS, 1992-2003).

Results

Gonad weight and gonad index

During the 20-day exposure period no mortality occurred among the test animals. Individual gonad weight was highly variable in all treatments (Table 1). Yet, gonad weight as well as gonad index were significantly reduced after phenanthrene exposure. In the solvent control, gonad weight was also reduced but not significantly different from controls. Somatic size (horizontal and vertical test diameters) and somatic weight were not different at the beginning and at the end of the experiment as well as between treatments.

Table 1: Growth parameters of *Psammechinus miliaris* in the different treatments. Standard deviations are in parentheses. Different letters in the same row indicate statistical significant differences between the treatments (control at day 0: n = 24, all other treatments: n = 30, one-way ANOVA, Tukey HSD, $p < 0.05$).

	days			
	0	20		
parameter / treatment	control	control	solvent control	phenanthrene
mean vertical test diameter [mm]	17.5^{a} (1.7)	17.4^{a} (1.9)	17.6^{a} (2.5)	17.4^{a} (2.8)
mean horizontal test diameter [mm]	30.9^{a} (3.2)	30.4^{a} (3.3)	30.6^{a} (3.9)	31.8^{a} (3.5)
mean somatic weight [g]	13.0^{a} (3.6)	12.4^{a} (2.9)	12.7^{a} (3.9)	13.5^{a} (3.8)
mean gonad weight [mg]	723^{a} (445)	712^{a} (384)	582^{a} (493)	390^{b} (257)
gonad index [%]	5.4^{a} (2.2)	5.8^{a} (2.2)	4.5^{a} (2.2)	2.9^{b} (1.9)

Alterations of the general gonad structure

In control animals, acini of the gonads were characterized by the presence of oocytes and nutritive phagocytes (NP) surrounded by a relatively thin multi-layered acinal wall (Figure 1 a). Oocytes at

different developmental stages were present with the oogonia and previtellogenic oocytes (Figure 1 b) preferably found close to the acinal wall and the vitellogenic oocytes (Figure 1 c) and mature ova (Figure 1 d) in the lumen of the acini. The occurrence and frequency of the different oogenic stages varied depending on the reproductive stage of the animal as well as in the different acini of each individual. The NPs formed a meshwork inside of the acini which was more or less visible depending on the reproductive stage of the animal and the treatment.

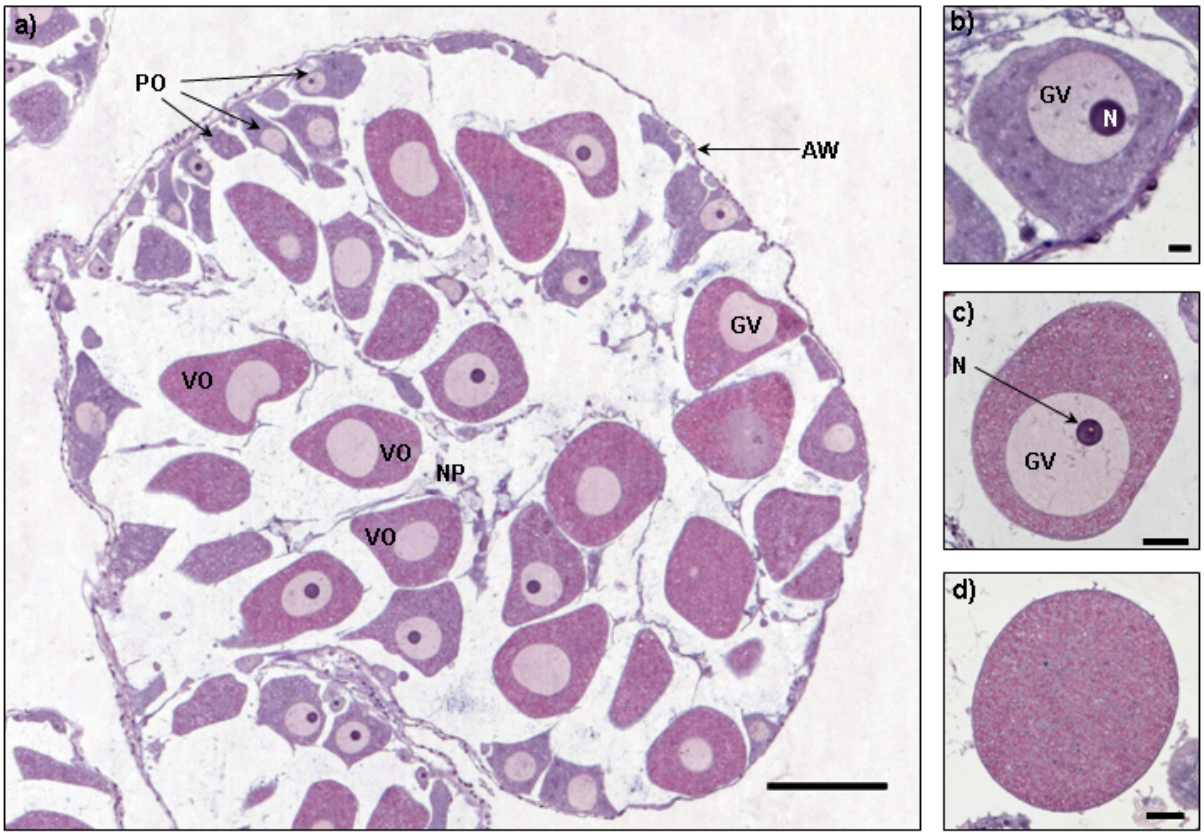

Figure 1: Ovarian sections of control animals of *Psammechinus miliaris* stained with Alcian Blue/PAS. a) Typical acinus of an ovary surrounded by a thin acinal wall. Early oocyte stages such as previtellogenic oocytes are found close to the acinal wall, whereas later oocyte stages such as vitellogenic oocytes are found in the lumen of the acinus. Note the empty spaces between the oocytes which result from spawning of oocytes. (bar = 100 µm) b) Previtellogenic oocyte of a control animal with a large germinal vesicle containing a big nucleolus (bar = 10 µm), c) vitellogenic oocyte showing PAS+ granules in the cytoplasm (bar = 20 µm), d) mature ova showing PAS+ granules in the cytoplasm and missing a germinal vesicle (bar 20 µm). AW = acinal wall, GV = germinal vesicle, N = nucleolus, NP = nutritive phagocytes, PO = previtellogenic oocyte, VO = vitellogenic oocyte.

At the beginning of the experiment (day 0) test animals (n = 10) were in the premature stage according to the classification of the reproductive stages by Byrne (1990) and Kelly (2000). The animals were partly spawned which was observed by empty spaces which had been occupied by oocytes before. At the end of the experiment (day 20), the sea urchins in the three treatments

were still, except of one control animal, in the premature stage and partially spawned (Figure 1 a). The one control animal was in the recovery period.

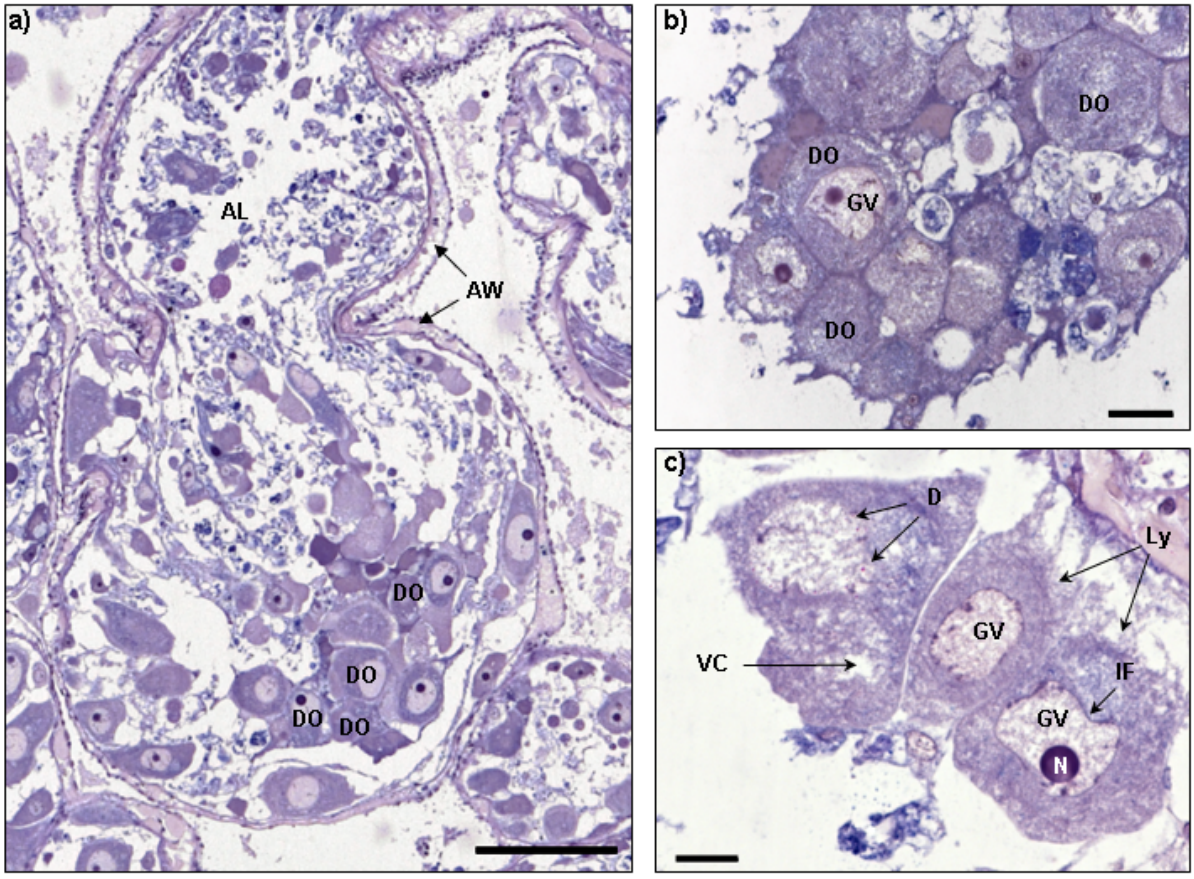

Figure 2: a) – c) Ovarian sections of phenanthrene-exposed *Psammechinus miliaris* stained with Alcian Blue/PAS. a) acinus showing severe disorganisation of the acinal structure with an aggregate of degenerating oocytes in the lower right and several small degenerating oocytes. Note the dilation of the acinal wall. (bar = 100 µm) b) aggregate of degenerating oocytes (bar = 20 µm) c) oocytes showing signs of degeneration with vacuolisation of the cytoplasm, cell lysis, infoldings of the nuclear membrane and disruption of the nuclear membrane (bar = 10 µm). AL = acinal lumen, AW = acinal wall, D = disruption of the nuclear membrane, DO = degenerating oocyte, GV = germinal vesicle, IF = infoldings of the nuclear membrane, Ly = cell lysis, N = nucleolus, NP = nutritive phagocytes, PO = previtellogenic oocyte, VC = vacuolisation of the cytoplasm.

In phenanthrene-exposed animals, the acinal structure was severely affected: early developmental stages of oocytes were no longer found close to the acinal wall but spread in the acinal lumen; the meshwork of NPs appeared disrupted and aggregates of degenerating previtellogenic oocytes were found in the acini (Figure 2 a, b). Control animals hardly showed any aggregates of degenerating oocytes while in phenanthrene-exposed animals these aggregates filled large areas of most of the acini. The areal density of these aggregates was significantly increased in phenanthrene-treated animals in comparison to the control and the solvent control groups (one-way ANOVA, $p = 0.006$,

Figure 3 a). Remarkably, less vitellogenic oocytes were found in the gonads of animals exposed to phenanthrene in comparison to controls.

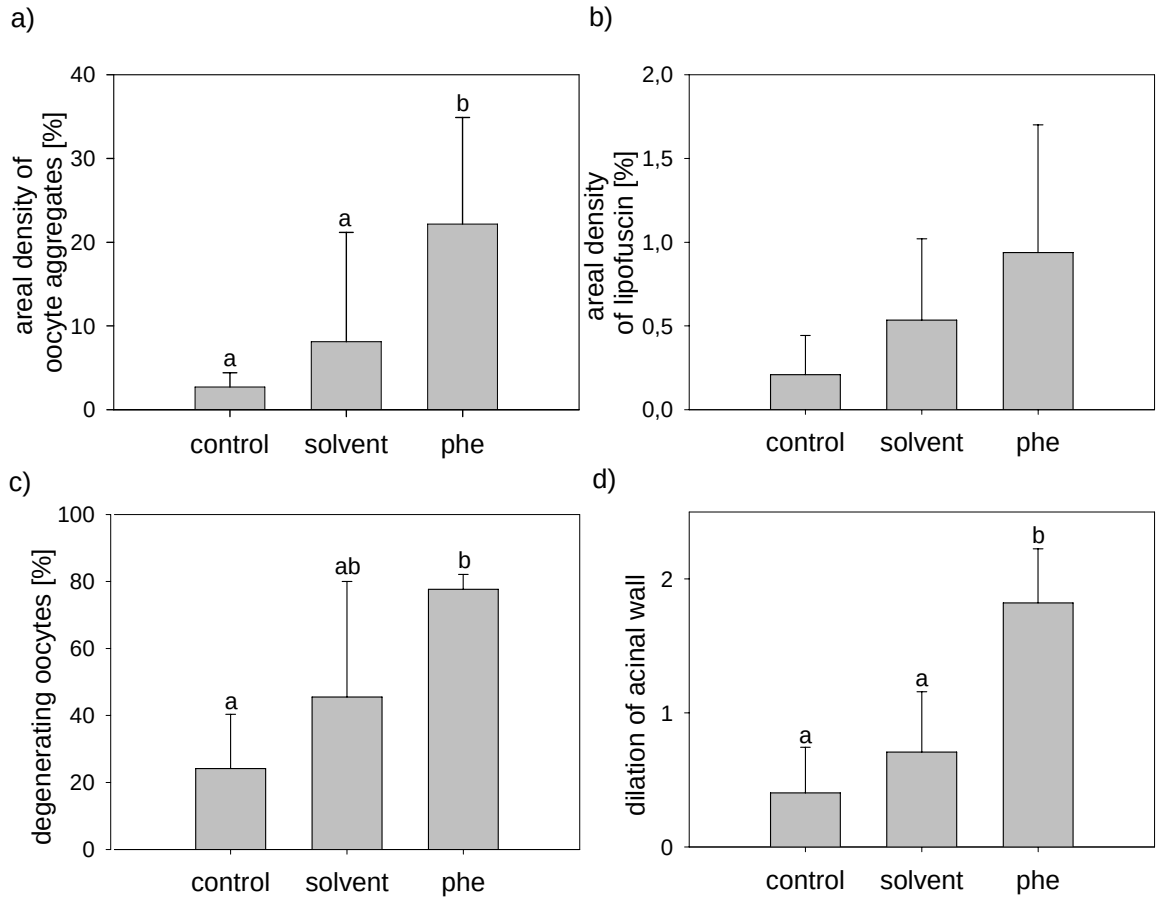

Figure 3: Histopathologic alterations depicted as bar charts with mean values and standard deviations. Different letter indicate significant differences ($p < 0.05$). a) areal density [%] of aggregates of degenerating oocytes (one-way ANOVA, Holm-Sidak, $p= 0.006$), b) areal density [%] of lipofuscin accumulation in the ovaries (one-way ANOVA), c) percentage of degenerating previtellogenic oocytes (Kruskal-Wallis, Dunn's test, $p = 0.01$), d) dilation of the acinal wall (one-way ANOVA, Holm-Sidak, $p < 0.01$).

Lipofuscin identified as irregularly shaped and heterogeneously textured blue granules in sections stained with the Schmorl's technique was found in the acini (Figure 4 a, b). High amounts of lipofuscin were localized in extracellular spaces in the acini and inside of NPs (Figure 4 a, b). In addition, lipofuscin was found in epithelial cells of the acinal wall and in conjunction with immune cells, the so called coelomocytes, outside of the acini (Figure 4 a). No lipofuscin was observed in oocytes. Indeed, early developmental stages of oocytes can hardly be identified in sections stained with the Schmorl's technique due to their small size and the lack of a counterstain. Lipofuscin deposition in oogonia and previtellogenic oocytes can, therefore, not be

excluded. Overall, lipofuscin content was increased but not significantly different in phenanthrene-treated animals (one-way ANOVA, p = 0.068, Figure 3 b).

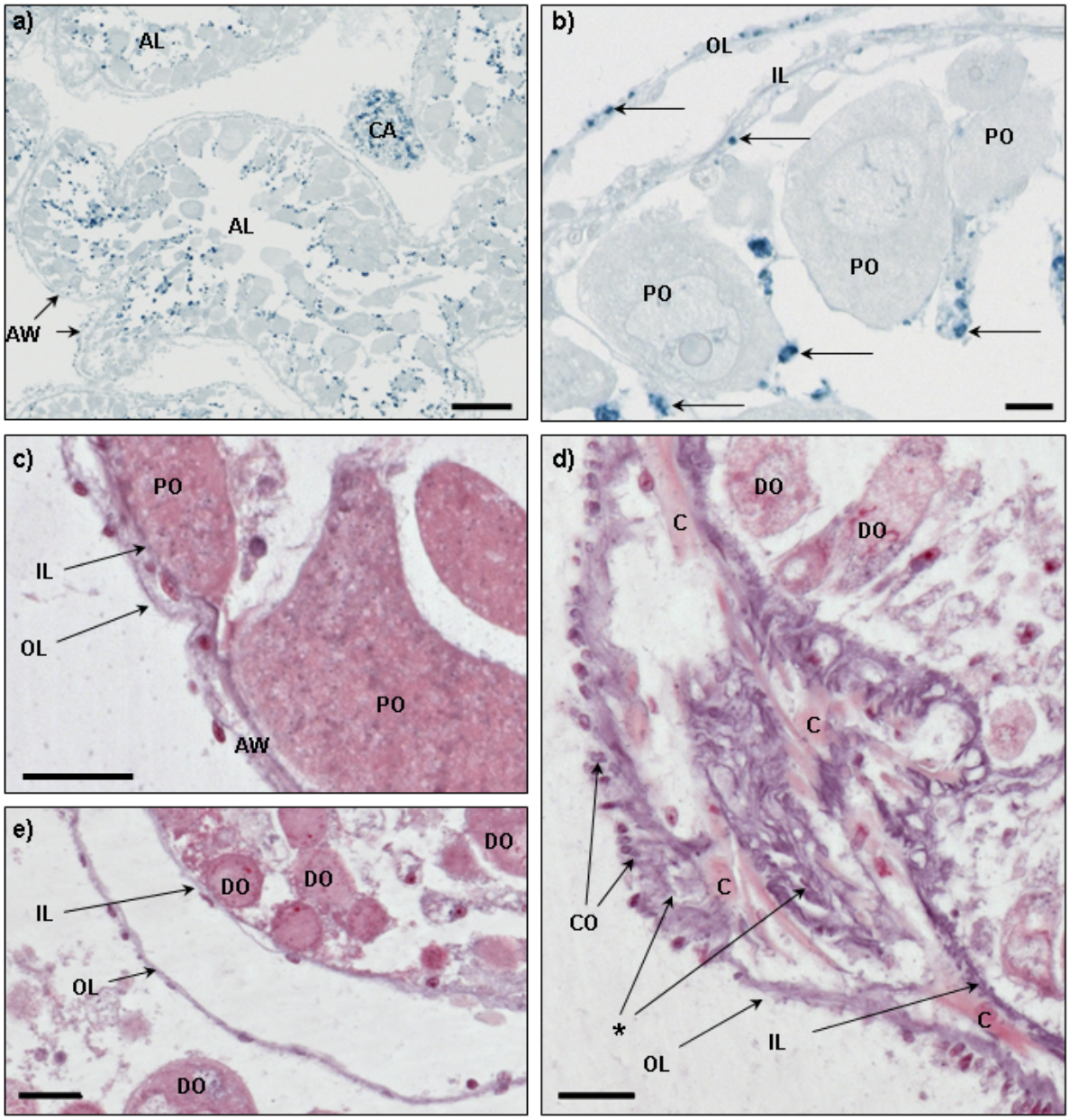

Figure 4: a) – b) Ovarian section of a phenanthrene-exposed individual stained with the Schmorl's method, a) overview taken at lower magnification showing high deposition of lipofuscin in the acini. Note the lipofuscin accumulation outside of the acini in conjunction with coelomocytes (bar = 100 µm), b) detail of a) with lipofuscin granules (arrows) (bar = 10 µm), c) – d) Ovary sections of *Psammechinus miliaris* stained with Picrosirius, c) Thin acinal wall of a control animal with the inner and outer epithelial layers (bar = 20 µm), d) Acinal wall of a phenanthrene-exposed animal showing dilation of the inner and outer epithelial layers concomitant with increased deposition of collagen and unknown basophilic material. Note the small cells at the outer epithelial layer which may be attacking coelomocytes (bar = 20 µm), e) Acinal wall of a phenanthrene-exposed animal showing dilation of the inner and outer epithelial layers (bar = 20 µm). AW = acinal wall, bas+ = basophilic material, C = collagen, CA = coelomocyte aggregate, CO = coelomocytes (?), DO = degenerating oocyte, GV = germinal vesicle, IL = inner epithelial layer, N = nucleolus, NP = nutritive phagocytes, OL = outer epithelial layer, PO = previtellogenic oocyte.

Effects on germ and somatic cells

In all treatments, oogonia, previtellogenic and vitellogenic oocytes as well as mature ova were found in the gonads. Previtellogenic oocytes are usually localized close to the acinal wall. Morphologically, they are characterized by a large germinal vesicle, a high nucleus to cytoplasmic ratio and their small size in comparison to later developmental stages. Their cytoplasm is stained blue in H&E and Alcian Blue/PAS (Figure 1 b). Vitellogenic oocytes, on the other hand, accumulate PAS+ material in the cytoplasm with ongoing maturation visible as pink granules in the Alcian Blue/PAS stained sections. Vitellogenic oocytes are larger and their nucleus to cytoplasmic ratio is reduced in comparison to previtellogenic oocytes (Figure 1 c). They also possess a large germinal vesicle which breaks down to form ripe ova (Figure 1 d) accumulating in the acinal lumen of mature animals.

We could not detect pathological changes of mature ova and vitellogenic oocytes while previtellogenic oocytes were often degenerating after maternal exposure to phenanthrene. Degenerating oocytes were characterized by fading and vacuolisation of the cytoplasm as well as cell lysis. Moreover, infoldings of the nuclear membrane of the GV occurred which was followed by disruption of the nuclear membrane (Figure 2 c). The nucleolus appeared to be normal. No pyknosis or karyomegaly of the GV was identified. In comparison to control samples, cell size of previtellogenic oocytes seemed to be reduced. Oogonia were not studied in detail due to their small size and the resultant difficulty in diagnosis of cellular alterations.

Degenerating previtellogenic oocytes identified by fading and vacuolisation of the cytoplasm, cell lysis and / or infoldings and disruption of the nuclear membrane were quantified in the gonadal tissue (Figure 3 c). In phenanthrene-treated animals 78 % of the previtellogenic oocytes were classified as degenerating based on the above mentioned criteria, whereas in controls 24 % degenerating previtellogenic oocytes were found. In the solvent control group, the frequency of degenerating oocytes was quite variable with a few animals exhibiting high amounts of degenerating oocytes. However, differences were only significant between the control group and phenanthrene-treated animals (one-way ANOVA, Holm-Sidak, $p = 0.01$).

As mentioned above, the meshwork of NPs was disrupted after exposure to phenanthrene. Moreover, in phenanthrene-exposed animals the NPs appeared to be more detached from each other and engaged in phagocytotic activity. No karyomegaly, nuclear polymorphism, or nuclear

inclusions were observed in NPs. Yolk was identified as globules of various shades of pink and blue in nutritive phagocytes in the Alcian Blue/PAS stain.

Alterations of the acinal wall

The acinal wall is usually composed of an inner and an outer layer of epithelial cells which are characterized by their basophilic nuclei. Depending on the plane of the tissue section at least one additional layer is visible being stained pink with H&E as well as with the Picrosirius method indicating the presence of collagen fibres (Figure 4 c).

In phenanthrene-exposed animals the two epithelial layers were often separate from each other (Figure 4 d, e). Often this dilation was concomitant with an increased formation of collagen between the epithelial layers and augmentation of basophilic material (Figure 4 d). Histological scoring shows that phenanthrene exposure resulted in significantly increased dilation of the acinal wall (one-way ANOVA, $p < 0.001$, Holm-Sidak, Figure 3 d). In solvent-treated samples dilation of the acinal wall occurred slightly more often than in control samples.

Discussion

It is well known that PAHs and oil elicit histopathological effects on gonads in marine invertebrates such as degeneration of germ cells and gonadal follicles, atrophy of gonadal tissue as well as inflammatory reactions (Lowe and Pipe 1986; Berthou et al. 1987; Cajaraville et al. 1990; Tay et al. 2003; Aarab et al. 2004; Ortiz-Zarragoitia and Cajaraville 2006). Indeed, the mentioned studies deal solely with molluscan species. To our knowledge the present study is the first one investigating the effects of PAH exposure on sea urchin gonads.

P. miliaris showed severe ovarian lesions after exposure to the sublethal concentration of phenanthrene. The gonad size and the gonad index of the sea urchins were significantly reduced indicating tissue atrophy. Similarly, Berthou et al. (1987) observed atrophy of the gonad concomitantly with a decrease in the gonad index in oysters in areas polluted by the Amoco Cadiz oil spill.

Strikingly, in *P. miliaris* exposed to phenanthrene the acinal structure was disorganised which was mainly due to aggregates of degenerating oocytes. This disorganisation makes a proper oogenesis unlikely since the migration of maturing oocytes towards the acinal lumen is hampered. Generally, degeneration of ovarian follicles is described as follicular atresia and stress such as

exposure to xenobiotics appears to increase the incidence of atretic follicles (Dietrich et al., 2009). Degenerating follicles were also observed in *Mytilus edulis* exposed to a mixture of North Sea oil, alkylphenols and PAHs. Exposure to 0.5 ppm North Sea oil alone, however, increased the size and number of ovarian follicles in the mussels (Aarab et al. 2004). In the present study, a few degenerating acini were also found in control animals which may resorb malfunctional oocytes to preserve normal gonadal function as known from atretic follicles in female fish (Dietrich et al. 2009).

Furthermore, phenanthrene exposure significantly increased degeneration of previtellogenic oocytes in the ovary of *P. miliaris*. PAHs and oil are known to increase the prevalence of oocyte degeneration or oocyte atresia in molluscs: Ortiz-Zarragoitia and Cajaraville (2006) found a higher volume density of atretic oocytes in mussels (*M. edulis*) exposed to 0.5 ppm North Sea oil and to a mixture of North Sea oil (0.5 ppm), alkylphenols (0.1 ppm) and extra polycyclic aromatic hydrocarbons (0.1 ppm) for three weeks. In *M. edulis* experimentally exposed to low (28 ppb) and high (128 ppb) doses of diesel oil for three months the volume of atretic oocytes was also significantly increased (Lowe and Pipe 1986). In the Baltic clam (*Macoma balthica*), necrosis of primary and secondary oocytes as well as ripe ova was reported after 28 days exposure to sediments highly contaminated with PAHs, polychlorinated biphenyls (PCBs), and heavy metals (Tay et al. 2003). Astonishingly, in clams exposed to sediment with a very high concentration of PAHs no ovarian lesions were detected (Tay et al. 2003).

In the present study, previtellogenic oocytes showed a reduction in size and vitellogenic oocytes showed a reduction in numbers in *P. miliaris* after maternal exposure to phenanthrene. It is unclear whether inhibition of oocyte growth and maturation by phenanthrene exposure is the underlying mechanism of these findings. The low number of vitellogenic oocytes may also be a result of spawning induced by exposure to phenanthrene since spawning is known to be accelerated in mussels (*Mytilus galloprovincialis*) exposed to high doses of oil (Cajaraville et al. 1992). However, no increased spawning events in the phenanthrene-treated group were observed during the experiment in comparison to controls. Regarding the previtellogenic oocytes, it could be speculated that their small size may, next to an inhibition of growth, result from degeneration and their resorption by NPs. Studying the effects of phenanthrene exposure at different times during oogenesis may clarify whether phenanthrene exposure inhibits growth and maturation of oocytes. Interestingly, in contrast to the severe alterations of previtellogenic oocytes, we found no adverse effects of phenanthrene on vitellogenic oocytes and mature eggs. It has to be considered

that later developmental stages of oocytes may be better protected against pollutant stress: eggs of sea urchins and sea stars are known to express low levels of efflux transporter genes (Hamdoun et al. 2004; Roepke et al. 2006). In sea stars, efflux transporter activities are known to increase during oocyte maturation (Roepke et al. 2006). Moreover, younger oocytes may be more sensitive to pollutants due to their higher surface to volume ratio. In addition, it has to be considered that release of affected gametes may result in underestimating the adverse effects of environmental pollutants on oocytes and eggs.

After exposure to phenanthrene we observed elevated but not statistically significant increased deposition of lipofuscin in the ovaries of *P. miliaris*. In several studies, more pronounced lipofuscin accumulation in fish liver and mussel digestive gland is associated with oxidative stress caused by pollution with oil and PAHs (Krishnakumar et al. 1994, 1997; Au et al. 1999; Au 2004). In the reproductive tissue of the sea urchin *Strongylocentrotus intermedius* inhabiting polluted areas in the Sea of Japan, lipofuscin accumulation was related to contamination with heavy metals (Vashchenko and Zhadan 1993; Vaschchenko et al. 2001; Vashchenko et al. 2001). As demonstrated in the present study, Vaschenko et al. (2001) and Vashchenko et al. (2001) also found lipofuscin deposition in NPs and in the hemal sinuses of the acinal walls. However, in contrast to our findings, the authors further identified lipofuscin accumulation in germ cells of *S. intermedius* (Vaschenko et al. 2001; Vashchenko et al. 2001). Indeed, preliminary studies with a wild population of *P. miliaris* indicate that lipofuscin accumulation in the reproductive tissue is dependent on the reproductive stage and the sex of the organism (Schäfer & Köhler unpublished). It has to be further investigated whether lipopigment deposition in reproductive tissue of sea urchins is a useful biomarker for pollutant stress.

The NPs showed increased phagocytic activity in the phenanthrene-treated animals. This may indicate early phagocytosis and resorption of gonadal and oocyte material as a result of improper ovarian and gamete development. Gamete resorption is a natural phenomenon in echinoderms that occurs in both sexes at the end of the spawning season (Kalachev and Reunov 2005) but it is also described in the sea urchin *S. intermedius* from polluted areas in the Sea of Japan (Vashchenko and Zhadan 1993). PAH-induced gamete resorption in gonads is also reported in the prosobranch *Littorina littorea* after short-term exposure to high concentrations of the PAH 1-napthol (Cajaraville et al. 1990).

The acinal wall of *P. miliaris* was severely affected by phenanthrene exposure showing dilation of the epithelial layers and increased formation of collagen. Furthermore, augmentation of basophilic structures occurred which can not be identified so far. The thickness of the gonadal wall of the sea urchin *Arbacia punctulata* is reported to change during the year (Palmer Wilson 1940). Increased deposition of collagen in conjunction with a thickened appearance of connective tissue, however, is generally known as fibrosis (Dietrich et al. 2009). In reproductive tissue of invertebrates fibrosis is described from gonadal neoplasms in mussels (Peters et al. 1994). And in the prosobranch *L. littorea* fibrosis was shown after short-term exposure to high concentrations of the PAH 1-napthol (Cajaraville et al. 1990). When immune cells are present simultaneously fibrosis is assumed to be a scarring process. In a few studies infiltration with immune cells have been described in invertebrates after exposure to oil and PAHs (Berthou et al. 1987; Cajaraville et al. 1990; Cajaraville et al. 1992). Indeed, a clear diagnosis of immune reactions in the present study was difficult since, to our knowledge, there is no detailed histological description of immune cells of sea urchins, the coelomocytes, *in situ*. In addition, coelomocytes had a similar appearance as epithelial cells of the acinal wall in *P. miliaris*.

The concentration of phenanthrene used in the present study is, in general, higher than levels of PAHs found in the marine environment: in seawater around England and Wales background concentrations of PAHs range from none detected to 10.7 µg L^{-1} total PAHs (Law et al. 1997). 'Safe' levels of phenanthrene for aquatic organisms set by several countries are equal or below 4.6 µg L^{-1} (Law et al. 1997). After the Exxon Valdez oil spill in Prince William Sound in the Northern Gulf of Alaska in 1989 where around 37 000 metric tons of crude oil had been released an average of 0.4 µg L^{-1} total petroleum hydrocarbon were measured at polluted sites (Boehm et al. 1990). However, after the Hebei Spirit incident at the West coast of Korea in July 2007 up to 17 mg L^{-1} and an average of 626 µg L^{-1} total petroleum hydrocarbons were measured (Shim 2008). Nonetheless, direct comparison of concentrations used in the present study and levels of PAHs or phenanthrene found in the marine environment in general or at point sources are difficult since combinations of PAHs may have different effects than single chemicals (Hylland 2006). Contamination of sea urchin habitats with PAHs, e.g. by oil spills or release of oil from oil platforms may have severe impacts on sea urchin populations. Daan & Mulder (1996), for example, have shown that the abundance of the echinoid *Echinocardium cordatum* was reduced close to a drilling station in the North Sea where oil-based drilling muds had been used. Following the Torrey Canyon oil spill in 1967, large numbers of dead *P. miliaris* where found in the vicinity

which may be attributed to the hydrocarbon exposure and the heavy spraying of hydrocarbon based dispersants in that area (Smith 1968).

In summary, 20 days exposure towards a sublethal concentration of phenanthrene resulted in severe ovarian lesions of *P. miliaris* which make a proper oogenesis unlikely. Strikingly, the acinal structure was disorganised which may be analogous to follicular atresia in other invertebrates and fish. Previtellogenic oocytes were severely affected while there were no obvious effects on vitellogenic oocytes and mature ova. For future studies, the usefulness of lipofuscin accumulation in gonads of sea urchins as a biomarker needs to be further investigated. Moreover, a detailed description of coelomocytes *in situ* is necessary to clearly identify immune reactions in sea urchin tissues and to compare histopathological effects of pollutants with studies on other invertebrate species.

Acknowledgements

This study was financed by the Alfred Wegener Institute for Polar and Marine Research within the Helmholtz Association. It is part of a PhD study conducted at the Jacobs University Bremen. The authors thank B. Hussel and the staff of the FS Mya for collecting the sea urchins. We are grateful to two anonymous reviewers for providing helpful comments on the manuscript.

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Histochemical alterations in gonads of the female sea urchin *Psammechinus miliaris* after exposure to the polycyclic aromatic hydrocarbon phenanthrene

Schäfer, S., Broeg, K., Köhler, A., *in preparation*.

Abstract

Exposure of adult organisms to environmental contaminants during gametogenesis is, in general, suspected to adversely affect developing gametes. Histochemical analyses of reproductive tissue may be useful for the identification of early changes in oocytes and eggs as they enable not only the measurement of cellular components and enzyme activities but also their simultaneous localisation in oocytes and eggs within the tissue. In a laboratory study, female sea urchins (*P. miliaris*) were exposed to 500 µg L^{-1} phenanthrene for 20 days and gonad samples were processed for histochemistry and analysed with the aid of computer assisted image analysis. Properties of oocyte lysosomes (areal density, size and numerical density) were not different in phenanthrene-treated oocytes and eggs compared to the control group. However, the density as well as the number of oocyte lysosomes were significantly decreased in the solvent control (0.01% acetone) compared to the control group. This implies that there must be an antagonistic effect of phenanthrene and its solvent on the lysosomal compartment in oocytes and eggs after maternal phenanthrene exposure. Following phenanthrene exposure, G6PDH activities as well as GSH contents were reduced but not significantly different from controls. In the case of G6PDH this may result from the low activity of this enzyme in unfertilized eggs. Activities of the detoxifying enzymes CYP450 reductase and ALDH could not be recorded using tetrazolium salt methods. The application of histochemical techniques for the detection of pollutant-mediated changes is discussed also in relation to the defense mechanisms of the developing oocytes against chemical stress.

Key words

sea urchin, oocyte lysosomes, G6PDH, GSH, histochemistry, phenanthrene

Introduction

Exposure of adult organisms towards environmental pollutants during gametogenesis may have adverse effects on developing gametes. In a parallel study, we found severe histopathological alterations in the reproductive tissue of the sea urchin *Psammechinus miliaris* after maternal exposure to phenanthrene. Interestingly, no effects on the later developmental stages of oocytes, namely secondary oocytes and mature eggs, could be detected by light microscopy. The aim of the present study was to identify potential early alterations in oocytes and eggs in the ovaries of *P. miliaris* after exposure to phenanthrene which have not been detected previously using light microscopy. For this purpose the following histochemical parameters were assessed in oocytes and eggs: size, volume density and numerical density of oocyte lysosomes or yolk platelets, GSH content and G6PDH activity.

Yolk is the main nutrient for the developing sea urchin embryo (Kavanu 1954). Due to the presence of several acid hydrolases yolk granules are considered as "lysosome-like" organelles (Pasteels 1973; Schuel et al. 1975; Pipe and Moore 1985). The hydrolytic enzymes within the yolk granules are believed to function in the mobilisation of the yolk material after fertilization (Schuel et al. 1975; Yamahama et al. 2003) and / or in the reabsorption of atretic oocytes (Pipe and Moore 1985; Carnevali et al. 2006). In the sea urchin *Strongylocentrotus purpuratus* the hydrolytic enzymes acid (nitrophenyl) phosphatase, α-L-fucosidase, N-acetyl glucosaminidase and N-acetyl galactosaminidase were localised in yolk platelets of unfertilised eggs (Schuel et al. 1975). Although the relationship between yolk granules and "classical" lysosomes still needs to be elucidated, they appear to be modified lysosomes or delayed lysosomes and are designated as the lysosomal system of oocytes and eggs (Cajaraville et al. 1991; Carnevali et al. 2006).

The lysosomal compartment in other cell types such as the digestive cells of molluscs and hepatic cells of fish are known to respond to a wide variety of environmental contaminants (reviewed in Au 2004; Viarengo et al. 2007). In molluscs, digestive cell lysosomes are able to accumulate xenobiotics such as PAHs which may result in reduction of lysosomal membrane stability and an increase in lysosomal enzyme activity as well as lysosomal size (Lowe et al. 1981; Au 2004). However, oocyte lysosomes in the mussel *Mytilus edulis* were smaller and much more numerous after exposure to high doses of petroleum hydrocarbons (Cajaraville et al. 1991).

Sea urchins possess a wide array of defense mechanisms against toxicant induced stress (Goldstone et al. 2006). An important protective role in xenobiotic metabolism plays reduced glutathione

(GSH, L-γ-glutamyl-L-cysteinyl-glycine) which is an ubiquitous tripeptide. Free radicals are detoxified either through direct interaction with GSH or enzymatic intervention via GSH peroxidase, producing the oxidized form of the thiol, glutathione disulfide (GSSG). Glutathione can also remove reactive intermediates and electrophiles through covalent adduct formation. GSH is supplied by reduction of GSSG by glutathione reductase (GSR), or by *de novo* synthesis via glutamate cysteine ligase and glutathione synthase. The GSH:GSSG ratio is the predominant factor in establishing and maintaining cellular redox potential which regulates various cellular functions. For many years the GSH pathways has been considered to be important for sea urchin development (Sakai and Dan 1959) and disruption of cellular redox status resulting from perturbation of GSH content is suspected to play a critical role in embryotoxicity (Wells and Winn 1996).

Furthermore, many enzymes are involved in cellular detoxification of environmental contaminants: CYP450 reductase is part of the cytochrome monooxygenase 450 system. CYP450 catalyzes the oxidation of xenobiotics such as PAHs (Fent 2003). CYP450 reductase, thereby, transfers the electrons derived from NADPH on CYP450 (Nelson and Cox 2001). Aldehyde dehydrogenase (ALDH), a phase II detoxifying enzyme, oxidizes highly reactive electrophilic aldehydes including compounds derived from the metabolism of a wide range of environmental chemicals (Goldstone et al. 2006). Glucose-6-phosphate dehydrogenase is the key enzyme of the pentose phosphate shunt which delivers not only DNA and RNA but also NADPH. NADPH is needed as reducing power for various detoxification pathways and thus, as defense against toxic injury. Cellular defense systems like enzymes (e.g. CYP 450 reductase) as well as non-enzymatic scavengers of reactive oxygen species (e.g. vitamins A and E and GSH) use NADPH as cofactor. GSH reductase, for example, maintains cellular GSH concentrations by catalysing the conversion of GSSG into reduced glutathione in the presence of NADPH (Wells and Winn 1996). During fertilization, NADPH in combination with O_2 is further utilized by NADPH oxidase to form H_20_2 (Rees et al. 1996) which in turn is used as substrate for ovoperoxidase for hardening of the fertilization membrane (Foerder and Shapiro 1977). G6PDH in hepatopancreatic cells in digestive glands of invertebrates such a molluscs and crustaceans as well as in the the liver of teleost fish is very sensitive to inhibition by exposure to environmental pollutants such as polycyclic aromatic hydrocarbons (e.g. Widdows et al. 1982; Teh et al. 1999; Winzer et al. 2002a).

Polycyclic aromatic hydrocarbons (PAHs) are lipophilic organic compounds which are ubiquitously found in the marine environment. They are classified as priority pollutants according

to several national and regional directives (ATSDR 1990; OSPAR 2007; HELCOM 2009). A major component of total PAH compounds in the marine waters and an important petroleum-source PAH is phenanthrene, a low molecular weight, 3-ring PAH.

In the present study, lysosomal structure (areal density, size, numerical density), enzyme activities and GSH content were measured in oocytes and eggs of sea urchin ovaries. For this purpose, cytochemical approaches in conjunction with stereological methods were applied with the aid of computer-assisted image analysis since they enable the direct measurements in tissue compartments or even in single cells such as oocytes and eggs.

Materials and Methods

Animal collection and experimental setup

The experiment was conducted in parallel to another study investigating histopathological alterations in ovaries of *Psammechinus miliaris* after exposure to phenanthrene (Schäfer and Köhler 2009). Adult *P. miliaris* were collected close to the Island of Sylt (Germany) in the northern Wadden Sea at 55° 02, 40N and 08° 27, 25E in May 2006. They were sorted by sex (Uhlig 1979) and transported to the laboratory in Bremerhaven. Female sea urchins of a minimum size of 20 mm horizontal test diameter were selected and acclimated to experimental conditions for 4 weeks. Sea urchins were exposed to 500 µg L^{-1} phenanthrene (predissolved in acetone), to 0.01 % acetone, as solvent control, or to sea water only (9 ± 0.5 °C, 33 ± 1 PSU, continuous aeration). Each treatment consisted of 3 replicate tanks (16 L glass aquaria) containing 10 sea urchins each. Water was renewed every third day and phenanthrene or solvent were added at the respective concentrations. Sea urchins were fed continuously with fresh spinach. Mortality of sea urchins was checked daily.

Sampling

Samples were taken at the day 20 of the exposure. The animals were dissected and the gonads were removed, weighed and immediately frozen in liquid nitrogen. Samples were stored at -80°C until further processing. Serial sections of 10 µm thickness were cut in a HM 500 OM microtome cryostat at -25°C and at constant speed.

Histochemical detection of acid phosphatase activity

Cryostat sections were incubated with 0.2 mg ml^{-1} Naphthol AS-BI phosphate in buffer (7% polypep and 3% NaCl in citric buffer, pH 4.5) at 37 °C for 15 min. Sections were rinsed twice with 3% NaCl solution at 37 °C and stained with 0.1% fast violet B salt in phosphate buffer (pH 7.4) at room temperature for 10 min. Then they were rinsed in tap water for 10 minutes and fixed in Baker's Formol Calcium (4% formaldehyde and 2% calcium acetate) for 15 min. The sections were rinsed again twice in distilled water and mounted in Kaiser's glycerol gelatine.

Analysis of enzyme activity based on tetrazolium salt methods

Histochemical detection of G6PDH activity was performed as described by van Noorden & Frederiks (1992) with slight modifications: 18 % PVA (w/v) was dissolved in 0.1 M phosphate buffer under continuous stirring at 60 °C. After cooling down to 37 °C, 10mM Glucose-6-phosphate, 5 mM sodium azide, 0.45 mM PMS, 5 mM NADP, 5 mM $MgCl_2$ were added. 5 mM TNBT was dissolved in equal volumes of ethanol and DMSO (2.5 mg in 100 µl ethanol and 100 µl DMSO for 5 ml incubation medium) first and then added to the medium. Cryostat sections were incubated in the dark at room temperature for 8 minutes. The reaction was stopped with 0.1 M phosphate buffer (pH 5.3) at 60 °C. Sections were left to dry and mounted in glycerol gelatine. For determination of unspecific formazan production control incubations without substrate and coenzyme were prepared in the same way.

Cryostat sections were incubated in a similar way for the demonstration of aldehyde dehydrogenase (ALDH) and cytochrome P450 (CYP450) reductase activity according to the method described by (Winzer et al. 2002b) with slight modifications (Table 1).

Table 1: Compounds of incubation media for histochemical assays based on TNBT reduction.

compounds / enzyme	G6PDH	ALDH	CYP 450 reductase
phosphate buffer	pH 7.4	pH 7.0	pH 7.4
substrate	glucose-6-phosphate (10 mM)	benzaldehyde (0.26 ml / ml acetone)	0.5 mM NADPH
coenzyme	5 mM NADP	5 mM NADP	
additional factors	0.45 PMS 5 mM sodium azide 5 mM $MgCl_2$	0.45 PMS 5 mM sodium azide	
control incubations	1) without substrate and coenzyme	1) without substrate and coenzyme	1) without substrate 2) 5mM NADP – substrate inhibition
incubation time [min.]	8	40	60

Analysis of reduced glutathione

Reduced glutathione was localized in oocytes by mercury orange (MO) fluorescence according to Asghar et al. (1975), de Jong et al. (2001) and Winzer et al. (2002b). Mercury orange (1-(4-chloromercuryphenoylazo)-2-napthol) forms complexes with sulfhydryl (SH) groups which are present in GSH and proteins. When short incubation periods are used, MO preferentially stains GSH (Asghar et al. 1975). Cryostat sections were incubated with ice-cold 5 mM mercury orange in DMSO for 5 minutes. The reaction was stopped by washing off the mercury orange with DMSO. The slides were left to dry and mounted in Euparal.

Image Analysis of chromogenic and fluorogenic assays

Cytophotometric analyses were performed by quantitative video microscopy with a light microscope (Axioscope, Zeiss) connected to a camera (AxioCam MrC) and linked to a computer with the KS 300 software (Zeiss).

Since acid phosphatase activity was homogeneously distributed in oocyte lysosomes the density, size and number of these organelles could be measured by histochemical detection of acid phosphatase activity and automated image analysis. Measurements were taken at 1000x magnification in secondary oocytes and mature eggs. Secondary oocytes were distinguished from eggs by the presence of the germinal vesicle. For contrast enhancement a green filter was used. Black and white images were taken and the percentual area of oocyte lysosomes in relation to the oocyte cytoplasmic area, the oocyte cytoplasmic area and the number of oocyte lysosomes were measured. Since closely attached lysosomes could not be discriminated from each other, the "binscrap" function of the software was used which identified agglomerated yolk platelets by their size. The number of yolk platelets could then be calculated by the software. Depending on the presence of the developmental stages of oocytes 6 to 8 samples were measured per treatment. The lysosomal density [area %], the mean lysosomal size [μm^2] as well as the number of oocyte lysosomes per cytoplasmic area [# μm^{-2}] were calculated. No correction for section thickness was done.

For G6PDH activity absorbance of formazan was measured in individual eggs at 400x magnification using monochromatic light of 580 nm. Concentrations of the reaction product were calculated using the Lambert-Beer law:

$$A = \varepsilon \cdot c \cdot d$$

with A being measured absorbance, ε being the extinction coefficient of the reaction end product at the specific wavelength (19.000 L mol^{-1} cm^{-1}), and d being the thickness of the sections (10 µm). It was taken into account that one molecule of formazan is formed by the conversion of two molecules of substrate.

Reduced glutathione was measured semi quantitatively as mercury orange fluorescence in individual eggs at 400x magnification. A filter set of 450-490 nm with a beamspliter at 510 nm and an emission wavelength of 520 nm was used. Absorbance was measured as grey values in individual eggs. Background fluorescence of the slides was subtracted from individual fluorescence values.

For G6PDH activity and GSH content, five eggs per sample (6 samples / treatment) were measured and the mean values were calculated. For all fluorogenic and chromogenic assays, individual oocytes and eggs were selected with the region of interest (ROI) function of the software.

Statistics

Statistical tests were run with SigmaStat 3.0.1 (SPSS). Residuals were tested for normality and variance homogeneity. Prior to analysis of lysosomal parameters, two solvent control samples had to be excluded from further analysis as they were identified as outliers according to the Grubb's test. Data on lysosomal parameters were analysed with a two-way ANOVA with the Holm-Sidak method as post-hoc test. Regarding the data for G6PDH activity and GSH levels, the power of the one-way ANOVA was lower than the desired power of 0.800 so that the data had to be analysed with a Kruskal-Wallis test and multiple comparisons as post-hoc tests. The significance level was set at $p < 0.05$.

Results

In the following, responses of oocyte lysosomes as well as enzyme activities and GSH content in oocytes and eggs following maternal phenanthrene exposure are described in detail.

Oocyte lysosomes

In figure 1, the lysosomal density, the mean lysosomal size and the lysosomal number in secondary oocytes and mature ova in the three treatments are presented. The lysosomal density in secondary oocytes and eggs was 7.4 (±1.6) [area %] (see Figure 1a).

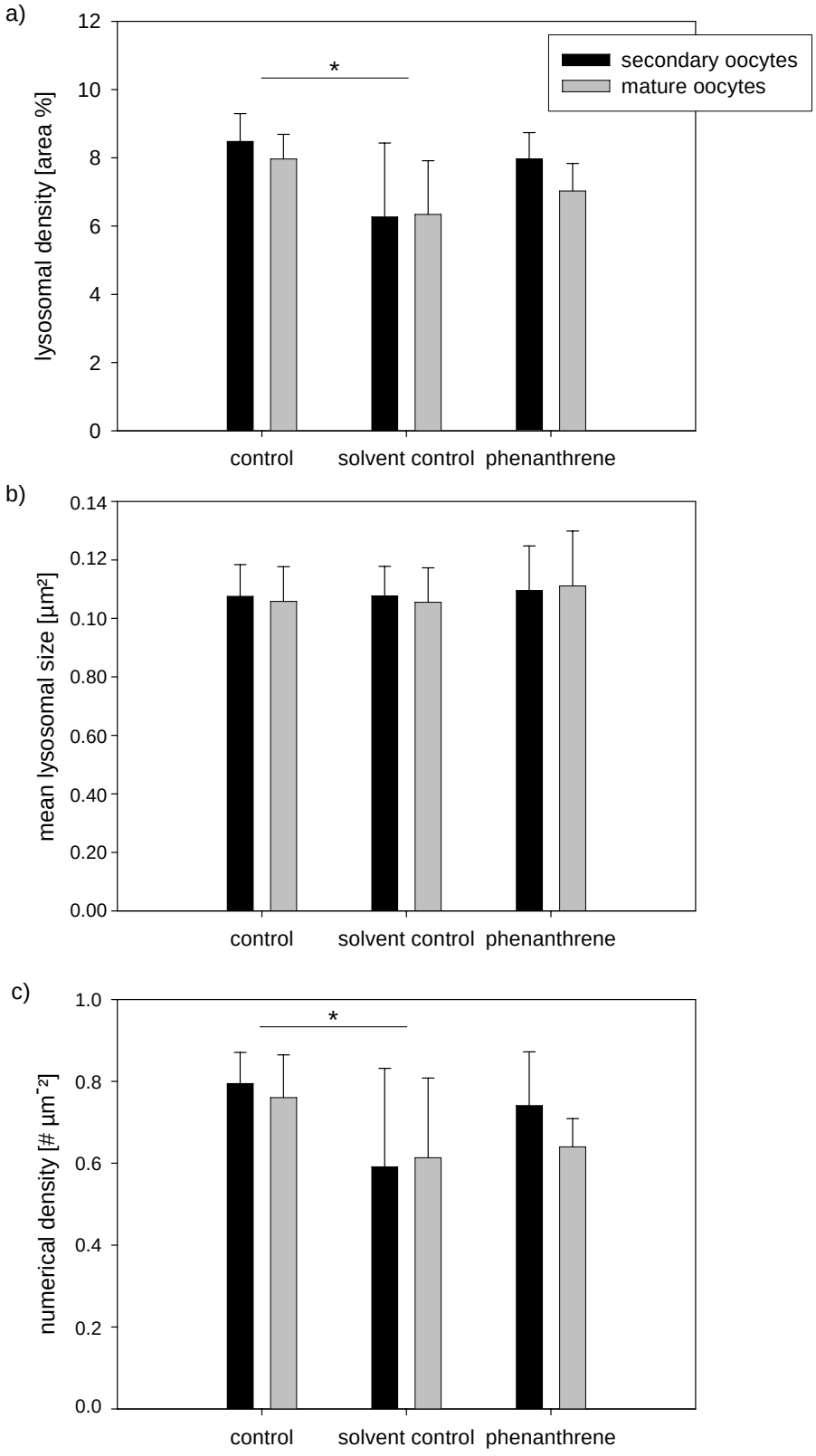

Figure 1: Properties of oocyte lysosomes in secondary oocytes (black bars) and mature ova (grey bars) in the different treatments. Asteriks indicate significant differences between treatments ($p < 0.05$). a) lysosomal density [area %] (2-way ANOVA, oocyte, $p = 0.154$, treatment, $p = 0.002$, interaction, $p = 0.620$, Holm-Sidak) b) mean lysosomal size [µm²] (2-way ANOVA, oocyte, $p = 0.854$, treatment, $p = 0.708$, interaction, $p = 0.918$) and c) numerical density [# μm^{-2}] (two-way ANOVA, oocyte, $p = 0.392$, treatment, $p = 0.025$, interaction, $p = 0.430$, Holm-Sidak).

No differences were found between the lysosomal density of secondary oocytes and eggs, whereas significant differences were evident between the control and the solvent control group (2-way ANOVA, oocytes: p = 0.154, treatment: p= 0.002, interaction: p = 0.620). The mean lysosomal size was 0.11 (±0.02) and did not differ between the oocyte stages and in the treatments (2-way ANOVA, oocytes: p =0.854, treatment: p = 0.780, interaction: p = 0.918). There are around 0.69 (± 0.16) [μm^{-2}] yolk granules per cytoplasmic area in the oocytes. In the solvent control the lysosomal number of oocytes and eggs was significantly reduced compared to controls with 0.61 (± 0.2) and 0.78 (± 0.09) [μm^{-2}], respectively (2-way ANOVA, oocytes: p = 0.392, treatment: p = 0.025, interaction: p = 0.430). No differences were found in lysosomal numbers between the phenanthrene-treated oocytes/eggs and the two other treatments.

G6PDH activity

The activity of G6PDH in mature ova was approximately 5 µmol G6P min^{-1} cm^{-1} (see Figure 2a). In the solvent control and the phenanthrene-treated animals, G6PDH activity in the eggs was decreased but not significantly different from controls (Kruskal-Wallis ANOVA on ranks, p > 0.095). ALDH and CYP450 reductase activity were with 0.02 µmol benzaldehyde min^{-1} cm^{-1} and 0.06 µmol NADPH min^{-1} cm^{-1}, respectively, close to the detection limit (data not presented).

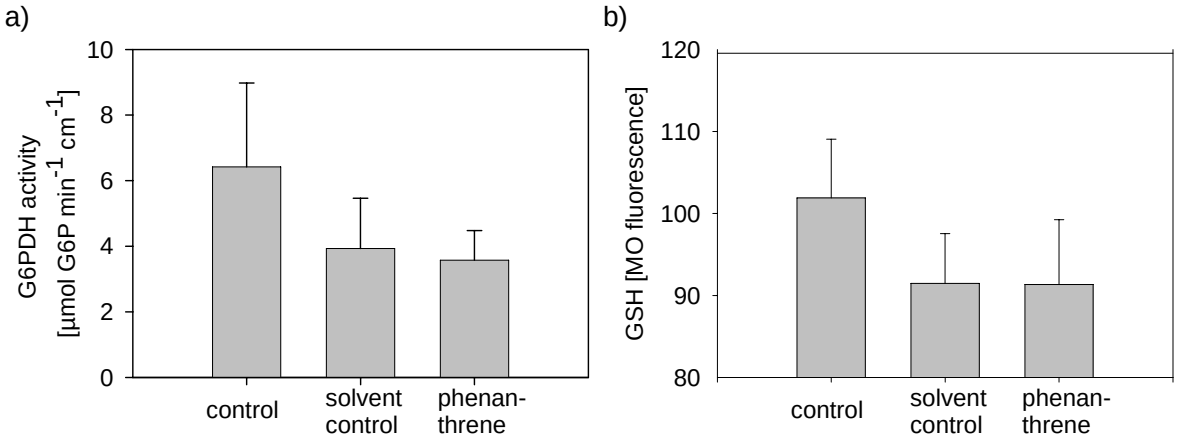

Figure 2: a) G6PDH activity and b) GSH content measured as mercury orange (MO) fluorescence in eggs of *P. miliaris* in the different treatments. No significant differences were found in G6PDH activity (Kruskal-Wallis ANOVA on ranks, p = 0.095) and GSH content (Kruskal Wallis ANOVA on ranks, p = 0.130) in the different treatments.

Reduced glutathione

Reduced glutathione measured as mercury orange fluorescence was reduced in the solvent control and the phenanthrene samples (Figure 2b). Indeed, no significant differences could be found (Kruskal-Wallis ANOVA on ranks, p > 0.130). In parallel to mercury orange fluorescence,

autofluorescence of oocytes was measured. Substraction of autofluorescence values from mercury orange fluorescence of the oocytes resulted in the same results as regarding mercury orange fluorescence. Autofluorescence of ova in the different treatment was not significantly different (Kruskal-Wallis, $p > 0.130$).

Discussion

In the present study, none of the parameters investigated (lysosomal properties, G6PDH activity, GSH) show a clear effect of phenanthrene exposure on oocytes and eggs of sea urchins after maternal exposure. However, the decrease in lysosomal density as well as lysosomal numerical density in oocytes and ova in the solvent control indicates that acetone impairs the integrity of the lysosomal membrane. In general, solvents are known to affect membrane integrity (Garbe and Yukawa 2001). Since we did not find significant differences in oocyte lysosomes between the phenanthrene treatment, which contains acetone as solvent, and the controls, the phenanthrene itself does not seem to have an additional synergistic effect on the lysosomal compartments in oocytes and eggs of *P. miliaris*. Rather, in the phenanthrene treatment the PAH acts antagonistically to its solvent on the oocyte lysosomes by increasing their areal as well as numerical density.

Lysosomes in the digestive gland of molluscs and in liver of fish are known to accumulate a wide range of contaminants such as PAHs which in turn may provoke significant alterations of these organelles. In the digestive gland of molluscs changes of the lysosomal compartment are usually associated with reduced stability of the lysosomal membranes and the enlargement of lysosomes (reviewed in Au 2004; Moore et al. 2004). In line with the present study, Lowe et al. (1981) found an enlargement of lysosomes and a decrease in the number of lysosomes in the digestive cells of the mussel *Mytilus edulis* after hydrocarbon (North Sea crude oil) treatment. In detail, lysosomal volume density and surface density was significantly increased after 34 and 103 days exposure to 30 µg L^{-1} aromatic hydrocarbons while lysosomal numerical density significantly increased after 103 days of exposure. Lysosomal surface to volume ratio was reduced but not significantly different from controls. However, in the mussel *Mytilus galloprovincialis* oocyte lysosomes were smaller and much more numerous after 21 days exposure to petroleum hydrocarbons so that the authors suggested that oocyte lysosomes differ from somatic cell lysosomes in respect to toxicant responses (Cajaraville et al. 1991).

We applied a similar approach as Cajaraville et al. (1991) for the measurement of oocyte lysosomes. Though, due to the small size of oocyte lysosomes the image analysis system we used was at its resolution limit (Christiane Schäfer, ZEISS, pers. communication). Therefore, we did intentionally not assess volumetric parameters (volume density, surface density, surface to volume ratio and numerical density) (Weibel et al. 1966; Lowe et al. 1981) but decided to calculate the size, numbers and densities of lysosomes in regard to the area of the respective oocyte or egg.

The effects of phenanthrene on lysosomal membranes have been investigated in a few studies: Ultrastructural studies in *M. edulis* have shown that phenanthrene can directly interact with the lipoprotein membrane of digestive lysosomes after exposure to 200 µg L^{-1} phenanthrene for only 22 hours (Nott et al. 1985). Similarly, in the snail *Littorina littorea* Pipe and Moore (1986) observed blebbing and gaps in the lysosomal membrane after treatment with 400 µg L^{-1} phenanthrene for three days. Structural changes in the lysosomal membrane of molluscs could be linked to a decrease of lysosmal membrane stability (Moore et al. 1985; Nott and Moore 1987). In another study, Einsporn and Köhler (2008) could also relate ultrastructural changes in the lysosomes to subcellular accumulation of phenanthrene inside of these organelles in the blue mussel (*Mytilus edulis*).

In the present study, oocyte lysosomes of *Psammechinus miliaris* were demonstrated by acid phosphatase activity. The same enzyme has already been used by Schuel et al. (1975) as a marker for oocyte lysosomes in the sea urchin *Strongylocentrotus purpuratus.* In *S. purpuratus* acid phosphatase and α-L-fucosidase were evenly distributed in all oocyte lysosomes. In contrast, the acid hydrolases N-acetyl glucosaminidase and N-acetyl galactosaminidase were only found in a smaller lysosomal subpopulation. We, therefore, assume that in our study the effects measured with the aid of acid phosphatase reflect changes in all types of lysosomes inside of the oocytes and eggs.

Reduced glutathione is known to be the main low molecular weight thiol in sea urchin eggs (Fahey et al. 1976). In eggs of the sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* total cellular glutathione is maintained at a highly reduced state with a GSH : GSSG ratio of 300 : 1 to 1000 : 1 (Fahey et al. 1976). The GSH pathways have long been considered to be important for sea urchin development (Sakai and Dan 1959). However, overall GSH and GSSG levels remain constant during fertilization and first cell division cycle indicating that they are not involved in controlling early development (Fahey et al. 1976). Gonadal tissues of marine invertebrates contain

very high GSH concentrations, particularly during peak reproductive periods. In oysters (*Crassostrea virginica*), embryos from parents with experimentally depleted GSH levels in the gonads were more susceptible to metal toxicity. Since gonads of oysters are simple follicles filled with gametes, gonadal GSH levels of parents are supposed to reflect the GSH levels of gametes (Ringwood and Conners 2000). In a field study, Ringwood et al. (2004) could further correlate parental GSH status of the digestive gland in *C. virginica* with susceptibility of embryos to metal toxicity. Indeed, no relationship between paternal GSH depletion and susceptibility of fertilization to metal toxicity as well as embryo development success were found (Ringwood and Conners 2000; Ringwood et al. 2004). PAHs are known to decrease GSH levels in cell lines as Winzer et al. (2002b) have shown for hepatocytes of the European flounder (*Platichthys flesus* L.) treated with the PAH benzo-[a]-pyrene. Nonetheless, we were not able to detect a significant effect of maternal phenanthrene exposure on GSH levels in eggs of sea urchins.

We exclude the possibility that mercury orange fluorescence is not appropriate for our question as this methodology has widely been used for the detection of GSH content in single cells (Winzer et al. 2002b) and in cryostat tissue sections (Asghar et al. 1975; Vukovic et al. 2000b; Beck et al. 2001; De Jong et al. 2001). And though GSH levels can only be determined semi quantitatively with MO fluorescence, Vukovic et al. (2000) have shown that GSH content in human tumor tissue determined with MO fluorescence strongly correlates with GSH concentration measured with HPLC when these methods were applied to serial cryostat sections. Increasing the number of replicates in future experiments may help to clarify if maternal exposure to environmental contaminants, in fact, does not affect GSH levels in eggs as shown in the present study.

G6PDH activity has been used as biomarker for contaminant exposure in marine fish (e.g. Van Noorden et al. 1997; Köhler and Van Noorden 1998; Köhler et al. 2004) and invertebrates (Widdows et al. 1982; Teh et al. 1999). In the Asian clam *Potamocorbula amurensis* sampled in the San Francisco Bay decreased G6PDH activity in the digestive gland diverticula was correlated with contamination by metals (Teh et al. 1999). In the mussel *Mytilus edulis* exposed to 30 µg L^{-1} hydrocarbons for 140 days, G6PDH activity was significantly reduced only in the gills and kidney while there were no significant changes in the digestive gland, mantle and adductor muscle (Widdows et al. 1982). Moreover, in hepatocytes of the European flounder (*Platichthys flesus* L.) G6PDH activity was significantly reduced after only one day exposure to 100 µM of the PAH benzo-[a]-pyrene (Winzer et al. 2002a).

However, in unfertilized sea urchin eggs pentose phosphate shunt activity, determined by G6PDH rather than the other NADPH-generating enzyme 6-phosphogluconate dehydrogenase (6PGDH), is very low (Swezey and Epel 1995). Only after fertilization calcium mediates the activation of NAD kinase which converts NAD into NADP (Epel 1978) followed by an up to 460-fold temporary increase in pentose phosphate activity (Swezey and Epel 1995). The rate of this pathway is probably regulated by redistribution of G6PDH between insoluble and soluble locations (Rees et al. 1996). Thus, in the present study G6PDH activity in oocytes and eggs is probably bound and inactive for the most part resulting in the low activities measured. Furthermore, the anaerobic metabolism of sea urchin gonads (Bookbinder and Shick 1986, Schäfer et al. unpublished data) may also not necessitate high activity of G6PDH for production of NADPH as NADPH is, amongst others, needed for reducing of reactive oxygen species which are primarily formed during oxidative phosphorylation (Turrens 2003). Pollutant-mediated effects on the activity of this enzyme may, therefore, be hardly detectable in oocytes and eggs of sea urchins before fertilization.

It remains unclear if oocytes and eggs of sea urchin possess CYP reductase and ALDH activity and if these enzymes are inducible by exposure to organic contaminants: CYP450 induction has been used as biomarker of exposure to organic contaminants in marine molluscs (Livingstone et al. 1985; Livingstone et al. 2000) and echinoderms (den Besten et al. 2001; Danis et al. 2006). The activity of CYP450 reductase needed for electron transfer within the monooxygenase system has been less often measured than CYP450 activity. Moreover, in the pyloric caeca of sea stars (*Asterias rubens*) NADPH-cytochrome c reductase activity were not changed four days after injection of different concentrations of the PAH benzo[a]pyrene (den Besten et al. 1993). Though, cytochemically measured NADPH-neotetrazolium reductase was elevated after 4 month exposure to low (29 ppb) and high (123 ppb) levels of hydrocarbons in the digestive gland of mussels (*Mytilus edulis*) and periwinkles (*Littorina littorea*) (Livingstone et al. 1985). Marine invertebrates are known to metabolise hydrocarbons more slowly than fish which is consistent with higher levels of total CYP450 and inducible CYP1A activity (Livingstone 1998). Moreover, reproductive tissues generally have lower levels of biotransformation enzymes than the liver (vertebrates) and tissues associated with processing of food (invertebrates) (Livingstone 1998). In gonads of echinoderms CYP450 activity was measured in the sea star *Asterias rubens* while it could not be demonstrated in the echinoid *Echinus esculentus* (den Besten 1998). Due to functional differences and its low inducibility by xenobiotics, the CYP450 system of echinoderms is considered to be

associated in the metabolism of steroids (den Besten 1998). Regarding ALDH, sea urchins are known to possess 5 ALDH1-like genes which are considered to be most important in detoxification (Goldstone et al. 2006). However, there is, to our knowledge, no other publication about ALDH activity in sea urchin eggs or gonads available.

In conclusion, the present study illustrates the difficulty to detect histochemical changes in oocytes and eggs after maternal exposure to a polycyclic aromatic hydrocarbon. Enzyme histochemistry did not reveal changes in eggs after exposure of adult organisms. We can not rule out that developing oocytes are, indeed, well protected against pollutant stress which would also be in line with our previous findings from a light microscopic study (Schäfer and Köhler 2009). Hamdoun et al. (2004) further have shown that sea urchins eggs have multidrug transporter-mediated efflux activity which is upregulated after fertilization and supposed to be involved in protection of the embryo from xenobiotics. We indirectly confirmed the activity of a group of these efflux transporters, the multidrug resistance associated proteins (MRPs), by using its inhibitor MK571 in combination with the calcium indicator dye Fura-2 as „xenobiotic" to record calcium signalling at fertilization in *P. miliaris* (Schäfer et al. 2009). Moreover, the low activity or even absence of enzymes (CYP 450 reductase, ALDH) involved in activation of chemicals may further protect oocytes from toxic reactive metabolites generated during biotransformation of chemicals. However, the results on oocyte lysosomes indicate that oocytes and eggs are, indeed, affected after maternal exposure to phenanthrene. In future studies, electron microscopy may help to clarify the responses of oocyte lysosomes in more detail and biochemical approaches may elucidate detoxification mechanisms of reproductive tissues. Furthermore, lower solvent concentrations are recommended in laboratory exposure studies to exclude any vehicle effects.

Acknowledgements

This study was financed by the Alfred Wegener Institute for Polar and Marine Research within the Helmholtz Association. We thank Birgit Hussel and the crew of the FS Mya for collecting the sea urchins. We are grateful to Christiane Schäfer from the Zeiss Company for her help with the image analysis software.

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Sex-specific biochemical responses and histological changes in gonads of sea urchins (*Psammechinus miliaris*) after exposure to phenanthrene

Schäfer, S., Abele, D., Weihe, E., Köhler, A., *in preparation*.

Abstract

High levels of the polycyclic aromatic hydrocarbon (PAH) phenanthrene are known to affect sea urchin ovaries, but effects on the male reproductive tissues have not yet been investigated. In the present study, sea urchins (*Psammechinus miliaris*) of both sexes were exposed to high (150 µg L^{-1}) and environmentally relevant (5 µg L^{-1}) concentrations of phenanthrene over 10 days. While food intake was significantly decreased following exposure to 150 µg L^{-1} phenanthrene, histological indices (lipofuscin accumulation, fibrosis, oocyte atresia), energetic status (energy charge, sum adenylates, AMP/ATP ratio) as well as ascorbate levels in the gonads showed either little or no effect upon phenanthrene exposure. However, biochemical as well as histological parameters generally differed between male and female animals. Neither formation of fluorescent ageing pigment lipofuscin, nor fibrosis were induced in the gonads on PAH exposure, but both degenerate processes were more intense in males than females. In addition, oocyte atresia was not increased following exposure to phenanthrene but was significantly elevated in individuals that had undergone untimely spawning. The energy charge (EC), a measure for the energetic status, ranged at values of around 0.2, reflecting sea urchin gonads to rely mostly on anaerobic energy production. Glycolysis is supposed to be up-regulated by high AMP/ATP ratios, which largely exceeded values of 1 in the sea urchin ovary and testes. Similar to the histological parameters, neither EC nor the AMP/ATP ratio were affected by toxicant treatment, but influenced by sex: Females had significantly higher EC levels but significantly lower AMP/ATP ratios than males, resulting from higher ATP concentration in ovaries than testes. Moreover, phenanthrene exposure had no effect on gonadal ascorbate concentrations, but levels were around 5 times higher in female than male *P. miliaris*. However, following exposure to 5 µg L^{-1} phenanthrene, the overall concentration of adenylates (ATP+ADP+AMP) as well as single adenylate (ATP, AMP, ADP) levels were, in general, lower compared to the other treatments, indicating that gonadal energy metabolism can become disturbed already at environmentally relevant concentrations of phenanthrene. Sex-specific differences in biochemical and histological parameters show that

ovaries are less susceptible to oxidative stress than testes which may be related to the different life spans, as well as functional differences of mitochondria in female and male gametes.

Key words

Sea urchin, gonad, lipofuscin, energy charge, phenanthrene

Introduction

Many environmental pollutants are known to impair reproductive health of animals (Haschek and Rousseaux 1998) which may be critical for population survival. Histopathological biomarkers in the reproductive tissue can be used for early detection of pollutant insult, as well as to understand the mode of action of chemicals on processes related to reproduction (Au et al. 2001; Dietrich et al. 2009). Sea urchins play a key role in marine benthic communities, and changes in sea urchin populations have led to disturbances in the community structure of coastal waters (Scheibling 1986; Steneck et al. 2002). *Psammechinus miliaris* is a relatively small echinoid, found in the Atlantic from Scandinavia to the Azores, including the North Sea and the Baltic (Boschma et al. 1932; Campbell 1977). In a recent study, severe histopathological alterations were observed in gonads of female *P. miliaris* following exposure to phenanthrene (Schäfer and Köhler, in press), a low molecular weight, 3-ring polycyclic aromatic hydrocarbon (PAH), and a major component of total PAH compounds in marine waters. PAHs are lipophilic organic compounds which are widespread in the marine environment. They are considered as priority substances by several European and regional directives, aiming at protecting aquatic environments from anthropogenic impact (HELCOM 2009; OSPAR 2007; European Commission 2008). Following exposure to 500 µg phenanthrene L^{-1} for 20 days, disorganisation of the gonadal structure, fibrosis, aggregates of degenerating oocytes, as well as high numbers of atretic oocytes were found (Schäfer and Köhler 2009). However, background concentrations of PAHs in seawater amount to only maximally 10.7 µg L^{-1} total PAHs (Law et al. 1997), and thus range at least 50-times lower than the test concentration in our previous study. Moreover, several countries set phenanthrene 'safe' levels for aquatic organisms equal or less than 4.6 µg L^{-1} (Law et al. 1997).

Histological and histochemical changes in gonadal tissues, which may be indicative for reproductive impairment, include increased lipofuscin accumulation, fibrosis as well as oocyte

atresia. Lipofuscin, also known as fluorescent age pigment, is an end product of protein and lipid peroxidation which accumulates as an ultimate consequence of oxidative stress (Au et al. 1999; Au 2004; Terman and Brunk 2004). In mussels and fish, contamination with PAHs has been associated with a more pronounced accumulation of lipofuscin in polluted groups compared to control groups (Krishnakumar et al. 1994; Krishnakumar et al. 1997; Au et al. 1999; Au 2004). Nevertheless, even 500 µg L^{-1} phenanthrene did not accelerate lipofuscin deposition in ovaries of the sea urchin *Psammechinus miliaris* (Schäfer and Köhler 2009). The second histopathological parameter, fibrosis, is often described to occur in reproductive tissues of fish after exposure to environmental contaminants (Dietrich et al. 2009). Fibrosis involves augmentation of collagen and more massive appearance of the connective tissue (Dietrich et al. 2009), and phenanthrene exposure increases fibrosis in sea urchin gonads (Schäfer and Köhler 2009). Another histopathological parameter, easy to record in females, is the percentage of atretic oocytes in the ovaries. Atresia is a degenerative and resorptive process of oocytes which may become pathologic after chemical exposure (Blazer 2002; Schäfer and Köhler 2009; Dietrich et al. 2009).

Next to histological investigations, biochemical analyses of reproductive tissue may be prognostic for impaired gametogenesis caused by exposure to environmental pollutants. The adenylate energy charge (EC) is a measure for the energetic state of an organism, a tissue or a cell and calculated as:

EC = (ATP + ADP/2) / (ATP + ADP + AMP).

Theoretically, EC varies from 0 to 1, but in natural systems maximal values of 0.8 to 0.95 are recorded. The energy charge is stabilized by adjusting the rate of ATP synthesis and consumption (Ataullakhanov and Vitvitsky 2002). Adverse environmental conditions such as the presence of toxic substances are generally accompanied by a decreasing EC. Therefore, Ivanovici (1980) proposed the use of EC as an index for environmental stress. Since the 1980s, EC values have been measured in laboratory and field studies of polluted populations, to assess sublethal effects of chemical compounds on aquatic organisms (Verschraegen et al. 1985; Marazza et al. 1996; Schill and Köhler 2004). Another parameter which can be measured biochemically is the non-enzymatic antioxidant ascorbate, scavenger of highly reactive superoxide anions and other free radicals.

Female and male individuals of the same species can differ with respect to their susceptibility to environmental contaminants. Higher incidence of liver cancer in female flounder (*Platichthys flesus* L.) has been associated with sex-specific differences in NADPH metabolism involved in xenobiotic biotransformation (Köhler and Van Noorden 2003). Furthermore, sex-related

differences of various organismal and cellular markers have been observed in stress responses of fish following exposure to environmental contaminants (Afonso et al. 2003; Vega-Lopez et al. 2007). In the sea urchin *Anthocidaris crassipina*, a higher sensitivity of males, assessed by reduced sperm motility and fertilization success of offspring, was found after chronic Cd^{2+} exposure (Au et al. 2001). Nacci et al. (2000) observed significantly decreased fecundity of female but not of male sea urchins (*Arbacia punctulata*) after exposure to the metal lead.

The present study aims at investigating the effects of an environmentally relevant concentration (5 µg L^{-1}) as well as a high dose (150 µg L^{-1}) of phenanthrene on gonads of sea urchins. Histological investigations were carried out in conjunction with biochemical measurements, to clarify whether female and male sea urchins are differently affected by exposure to phenanthrene.

Materials and Methods

Animal collection, acclimation and experimental set up

Adult *P. miliaris* were collected with a beam trawl in the List Tidal Basin in the northern Wadden Sea (Germany) at 55° 02' 40N and 08° 27' 25E in December 2006. They were sorted by sex according to the appearance of their gonadopores following Uhlig (1979) and transported to the laboratory in Bremerhaven. Sea urchins were kept in aquaria in a small recirculation system at a water temperature of 6 °C and fed with *Fucus sp.* For induction of gametogenesis, winter conditions of the natural habitat were simulated by decreasing water temperatures as well as photoperiod gradually to 3 °C and 8.8:15.2 hours light:dark cycle, respectively, and increased again to 6°C and 11.7:12.3 light:dark in March 2007. Prior to the start of the experiment, sea urchins were transferred into flasks containing 1.5 L sea water (4 test animals per flask) and acclimated for 3 to 4 days. Sea urchins were exposed to either 5 µg L^{-1} phenanthrene, 150 µg L^{-1} phenanthrene (in each case phenanthrene was predissolved in acetone), to seawater plus 3 ppm acetone as solvent control, or to sea water only (8.6 ± 0.6 °C, 32.5 ± 0.1 PSU, pH 8.1 ± 0.1, continuous aeration). Each treatment consisted of three replicate flasks per sex (12 animals x sex x treatment). Sea urchins were fed once at the beginning of the experiment with fresh macroalgae (*Fucus serratus*). Wet weight of *F. serratus* leftovers at the end of the experiment was recorded in each flask to calculate the net food intake as mg^{-1} day^{-1}. Water was renewed daily and phenanthrene or solvent only added at the respective concentrations.

Sampling

Samples were taken after ten days of exposure. Animals were weighed (± 0.01g) and horizontal test diameters (HTD) (± 1 cm) were measured using callipers. The animals were dissected; the gonads were removed and weighed (± 1 mg). The gonad index (GI) was calculated as the wet weight of the gonad divided by the total somatic wet weight and expressed as percentage.

Histology

For histopathology, six samples per treatment and sex were taken and immediately fixed in Baker's Formol Calcium (4% formaldehyde and 2% calcium acetate) over night. On the next day, the samples were transferred to gum sucrose (30% sucrose and 1% gum arabicum) and stored at 4°C until further processing. Histological samples were dehydrated with acetone and embedded in methacrylate as described in detail elsewhere (Köhler 2004; Schäfer and Köhler 2009). Serial sections of 2 µm thickness were cut using a HM LEICA RM 2145 microtome and stained with the Alcian Blue PAS technique for general tissue morphology, as well as the Schmorl's procedure for lipofuscin. The Alcian Blue PAS technique was used instead of the conventional hematoxylin and eosin (H&E) staining, since it was previously shown to function optimally for gonadal tissues (Schäfer and Köhler 2009). Briefly, sections are stained with Alcian Blue according to Lendrum et al. (1972). Sections are placed overnight in aldehyde blocking solution (2% sodium chlorite in 6 % acetic acid) and stained with PAS (periodic acid Schiff's reagent) as described by (Schäfer and Köhler 2009). For the Schmorl's procedure, sections were stained with ferric chloride / potassium ferricyanide solution as described by (Schäfer and Köhler 2009). This technique results in blue staining of lipofuscin granules.

Histological sections were viewed with a microscope (Axioscope, Zeiss, Germany). Reproductive stage of each animal was determined according to the classification for sea urchins by Byrne (1990) and Kelly (2000). Lipofuscin was quantified as areal density [%] in cross sections of gonadal tissue with the aid of computer-assisted image analysis at 100x magnification (Schäfer and Köhler 2009). Fibrosis and untimely spawning were semi quantitatively assessed. For determination of fibrosis, in each sample 5 randomly chosen areas were graded at 100x magnification according to a scaling scheme from 0 (no change) to 3 (severe alteration). Premature sea urchins may spontaneously shed part of their gametes (Spirlet et al. 1998) which can be recognized by vacated spaces in the acini previously occupied by maturing gametes. Untimely spawning was graded in each individual at 100x magnification according to a scaling scheme from 0 (no spawning) to 2 (severe spawning). Atretic oocytes and eggs identified by fading and vacuolisation of the

cytoplasm, cell lysis and disruption of the nuclear membrane were counted at 600x magnification. Randomly selected oocytes and eggs of 100 were counted per sample and the data are expressed as percentage.

Tissue metabolites

Simultaneous analysis of EC parameters (ATP, ADP, AMP) and tissue ascorbate concentrations were analysed by HPLC according to Lazzarino et al. (2003). At least eight samples per treatment and sex were taken and ground in liquid nitrogen. Preparation of extracts and HPLC procedure have been documented previously by Weihe and Abele (2008). Ground gonad tissue was homogenised with a micropistill with ice-cold, nitrogen-saturated precipitation solution (CH_3CN [Acetonitril] + 10 mM KH_2PO_4, at a ratio of 3:1, pH 7.4) at a 1:10 (w:v) tissue to medium ratio. The precipitation solution was prepared weekly, and pH was checked daily. The homogenate was centrifuged at 20 690 x g for 10 min at 4°C, and the clear supernatants were stored on ice. Pellets were supplemented with 1 ml of the precipitation solution and resuspended for several seconds using an ultraturax, centrifuged again as above, and the supernatants combined. This extract was washed with the double volume of chloroform (10 s vortexed with HPLC grade CH_3Cl) and centrifuged as above. The upper aqueous phase, containing the water-soluble low molecular weight compounds, was collected and washed again twice with chloroform. Supernatants were then stored at -80°C until measurement.

Samples were separated on a Beckman HPLC using a Kromasil 250 x 4.6 mm, 5 µm particle size column (Eka Chemicals, AB, Bohus, Sweden) and its own guard column. Injection volume was 50 µl of extract. In some samples, sea urchins had relatively small gonads and only small tissue samples could be processed, resulting in low extraction volumes which had to be diluted with 10 mM KH_2PO_4, to obtain adequate extraction volumes. HPLC conditions (solvents, gradient, flow rate, detection) were applied as described in Lazzarino et al. (2003). AMP, ADP, ATP standards were purchased from Sigma.

Metabolite concentrations were assessed using Karat Software 7.0. Energy charge (EC) after Atkinson (cf. Ataullakhanov and Vitvitsky 2002) was calculated as

$$EC = (ATP + ADP/2) / (ATP + ADP + AMP).$$

The amount of total adenylates was calculated as

$$\text{Total adenylate} = ATP + ADP + AMP.$$

Statistics

Since treatment and sex may affect behaviour (food intake), somatic parameters as well as biochemical composition of gonads (adenylates, ascorbate), data were analysed by means of a 2-way ANOVA with interactive effect and a Fisher LSD as a post hoc test. Gonad index and gonad weight were ln- and log-transformed, respectively, prior analysis, to obtain normal distribution of data. Since lipofuscin content and fibrosis were not normally distributed, they were analysed by non-parametric methods. The effects of treatment on lipofuscin content and fibrosis were tested using a Kruskal Wallis ANOVA on ranks, whereas the effects of sex on both parameters were tested using a Mann Whitney rank sum test. For oocyte atresia the effects of treatment and spawning were analysed using one-way ANOVA and Fisher LSD post hoc test. The significance level was set as $p < 0.05$.

Results

Food intake, growth and gonad index

Food intake of *P. miliaris* was significantly reduced in sea urchins exposed to 150 µg phenanthrene L^{-1} (Table 1). No sex difference was observed between food intake in *P. miliaris* (2-way ANOVA, treatment $p < 0.031$, sex $p = 0.852$, interaction $p = 0.313$, Tukey HSD, $p < 0.05$).

Table 1: Food intake measured as mg *F. serratus* wet weight day^{-1}. Mean net food intake of three replicate glass vessels were calculated (n = 3). Different letters indicate significant differences between treatments (2-way ANOVA, Tukey HSD, $p < 0.05$).

treatment	sex	food intake [mg wet weight day^{-1}]
control	f	202 (56) [a]
control	m	461 (152) [a]
solvent control	f	182 (104) [a]
solvent control	m	162 (95) [a]
5 µg L^{-1} phenanthrene	f	188 (100) [a]
5 µg L^{-1} phenanthrene	m	195 (71) [a]
150 µg L^{-1} phenanthrene	f	113 (87) [b]
150 µg L^{-1} phenanthrene	m	58 (76) [b]

Somatic size (HTD) and somatic weight did not differ between treatments or sexes (HTD: sex p = 0.580, treatment p = 0.720, sex x treatment p = 0.976; somatic weight: sex p = 0.757, treatment p = 0.528, sex x treatment p = 0.992, Table 1). However, in all samples males had significantly less gonad weight compared to females (p = 0.023) with 577 ± 417 mg vs. 888 ± 688 mg, respectively. Furthermore, males had significantly lower gonad indices than females (p = 0.006) with 4.2 ± 2.6 % and 6.3 ± 3.4 %, respectively. Gonad weight was significantly reduced in phenanthrene-treated animals (5 µg L^{-1} and 150 µg L^{-1}) compared to the control group (p = 0.046), but gonad index did not differ between treatments (p = 0.066). No interaction of sex and treatment was recorded with respect to either gonad weight or gonad index effect (gonad weight: p = 0.199, GI: p = 0.176).

Table 2: Growth parameters of *P. miliaris* in the different treatments. F = females, m = males. VTD = vertical test diameter, HTD = horizontal test diameter, GI = gonad index. Presented are mean values with standard deviations in parentheses. Different letters in the same column indicate significant differences between treatment and / or sex (2-way ANOVA, Tukey HSD, $p < 0.05$).

treatment	sex	n	HTD [mm]	somatic weight [g]	gonad weight [mg]	GI [%]
control	f	11	30.5 (2.8)	13.5 (3.7)	1079 (706) ^{ac}	7.7 (3.5) ^{a}
control	m	12	31.3 (2.9)	14.0 (3.1)	712 (348) ^{bc}	5.3 (2.9) ^{b}
solvent control	f	12	30.9 (4.7)	15.0 (6.3)	1096 (806) ^{acd}	6.8 (3.1) ^{a}
solvent control	m	12	31.2 (4.0)	14.8 (5.2)	448 (164) ^{bcd}	3.3 (1.5) ^{b}
5 µg L^{-1} phenanthrene	f	10	29.4 (4.4)	12.6 (4.9)	622 (606) ^{ad}	5.1 (3.7) ^{a}
5 µg L^{-1} phenanthrene	m	12	30.3 (3.7)	13.1 (3.9)	731 (633) ^{bd}	5.0 (3.3) ^{b}
150 µg L^{-1} phenanthrene	f	11	30.6 (3.7)	13.6 (4.6)	710 (544) ^{ad}	5.4 (3.2) ^{a}
150 µg L^{-1} phenanthrene	m	12	30.6 (4.9)	14.0 (5.6)	416 (253) ^{bd}	3.3 (2.2) ^{b}

Histology

Except for one female, sea urchins were in the premature stage. Premature sea urchins are characterized by the presence of gametes in all stages of development. Mature gametes, eggs and spermatozoa, accumulate in the lumen of the gonadal acini, whereas the nutritive phagocytes are displaced to the acinal periphery (e.g. Byrne 1990; Kelly 2000). One female was partially spawned which became evident from large areas of vacant space between relict oocytes. This animal had been treated with 5 µg phenanthrene L^{-1}. As reproductive stage affects histocytological parameters

such as lipofuscin content and fibrosis in sea urchin gonads (Schäfer & Köhler, unpublished), the partially spawned female was excluded from the following analyses.

In all groups, lipofuscin content measured as areal density in gonadal cross sections was significantly increased in males compared to females with 1.716 ± 1.447 % and 0.417 ± 0.705 %, respectively (Mann Whitney Rank sum test, $p < 0.001$, Figure 1a). Treatment had no effect on lipofuscin content – neither in both sexes taken together (Kruskal Wallis ANOVA on ranks $p = 0.682$), nor within either sex if males and females were considered separately (females: Kruskal-Wallis ANOVA on ranks $p = 0.282$, males: Kruskal-Wallis ANOVA on ranks $p = 0.572$). Next to lipofuscin, fibrosis was significantly increased in males compared to females (Mann-Whitney Rank sum test, $p = 0.011$, Figure 1b). In females, fibrosis was elevated in phenanthrene-treated animals, but differences were not significant (Kruskal-Wallis ANOVA on ranks $p = 0.059$). In males as well as in both sexes taken together, occurrence of fibrosis did not differ between treatments (males: Kruskal-Wallis ANOVA on ranks $p = 0.225$, both sexes: Kruskal-Wallis ANOVA on ranks $p = 0.673$).

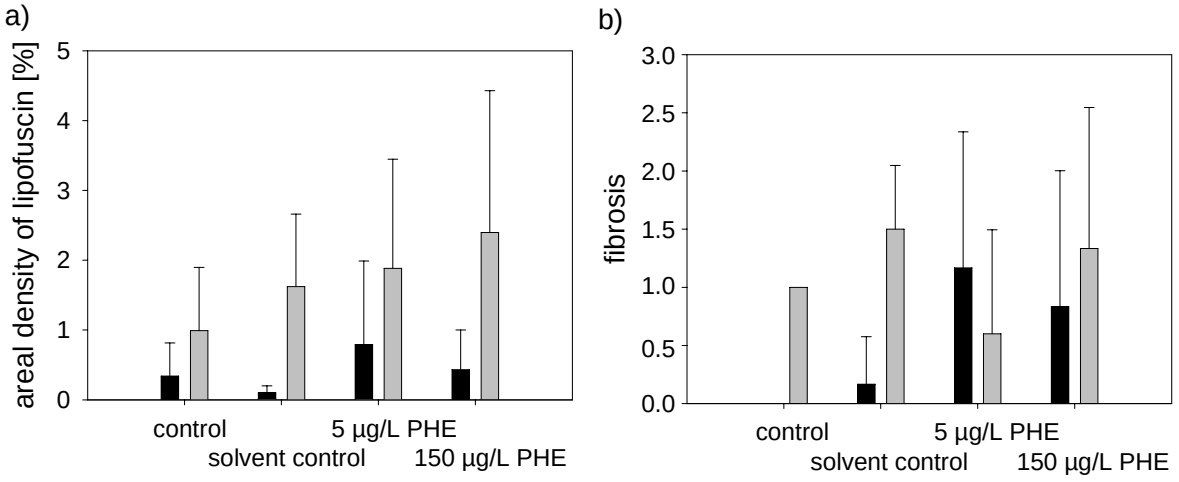

Figure 1: Histochemical and histopathological changes in gonads of female (black bars) and male (grey bars) *P. miliaris*. a) Lipofuscin content measured as areal density. Differences in sex are significant (Mann-Whitney Rank sum test, $p < 0.001$) while treatment has no effect on lipofuscin accumulation neither in males (Kruskal Wallis ANOVA on ranks, $p = 0.572$) nor in females (Kruskal Wallis ANOVA on ranks, $p = 0.282$). b) Fibrosis semi quantitatively determined also differed significantly in both sexes (Mann-Whitney Rank sum test, $p = 0.011$) while no effect of treatment can be observed (females: Kruskal-Wallis ANOVa, $p = 0.059$, males: Kruskal-Wallis ANOVA, $p = 0.225$).

As spawning may be induced by exposure to chemicals (Cajaraville et al. 1992) spawning was semi quantitatively recorded in sea urchin gonads. However, phenanthrene exposure did not result in

the untimely release of gametes (Kruskal-Wallis ANOVA on ranks, p = 0.068). Furthermore, treatment had no effect on the percentage of atretic oocytes (one-way ANOVA, p = 0.257). However, in all treatments (controls and phenanthrene exposed) spontaneous spawning events significantly increased the incidence of atretic oocytes (one-way ANOVA, p < 0.001, Fisher LSD, p < 0.05, Figure 2). Animals that had released many gametes - visible by large areas of vacant space in the gonads - had the highest percentage of atretic oocytes. The lowest numbers of atretic oocytes were found in females that had not yet released gametes. No other tissue alterations, such as disorganisation of the acinal structure, aggregation of degenerating oocytes, inflammatory reactions or changes in nutritive phagocytes were observed in the gonads.

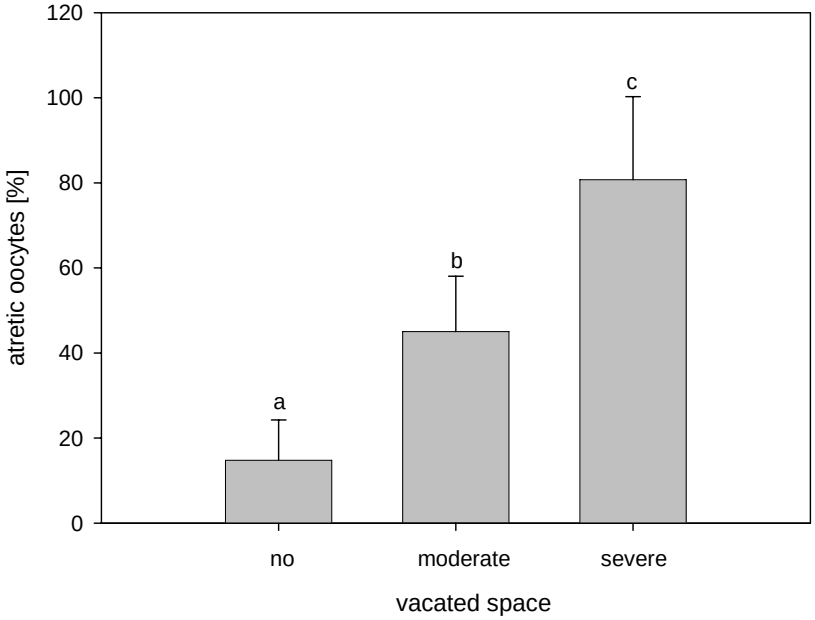

Figure 2: Percentage of atretic oocytes in ovaries of *P. milaris* in dependence of spawning. Animals that had underwent severe spawning events visible as large areas of space vacated by shed oocytes and eggs had significantly increased numbers of atretic oocytes (one-way ANOVA, p < 0.001, Fisher LSD, p < 0.05).

Parameters of energy charge and ascorbate concentrations

Adenylate concentrations differed very little between treatments, but were significantly different in female and male sea urchin (Figure 3). Moreover, metabolite concentrations were highly variable in all samples (compare Table 2). Energy charge (EC) (Figure 3a) was 0.25 ± 0.19 in females and significantly higher than in males which had an EC of only 0.15 ± 0.18. No differences were found in the EC between treatments (2-way ANOVA, sex: p < 0.001, treatment: p = 0.188,

interaction: p = 0.600). The sum of the adenylates (Figure 3b) were 1.5 times higher in females (528 ± 311 nmol g^{-1} fresh weight) than in males with only 342 ± 287 nmol g^{-1} fresh weight.

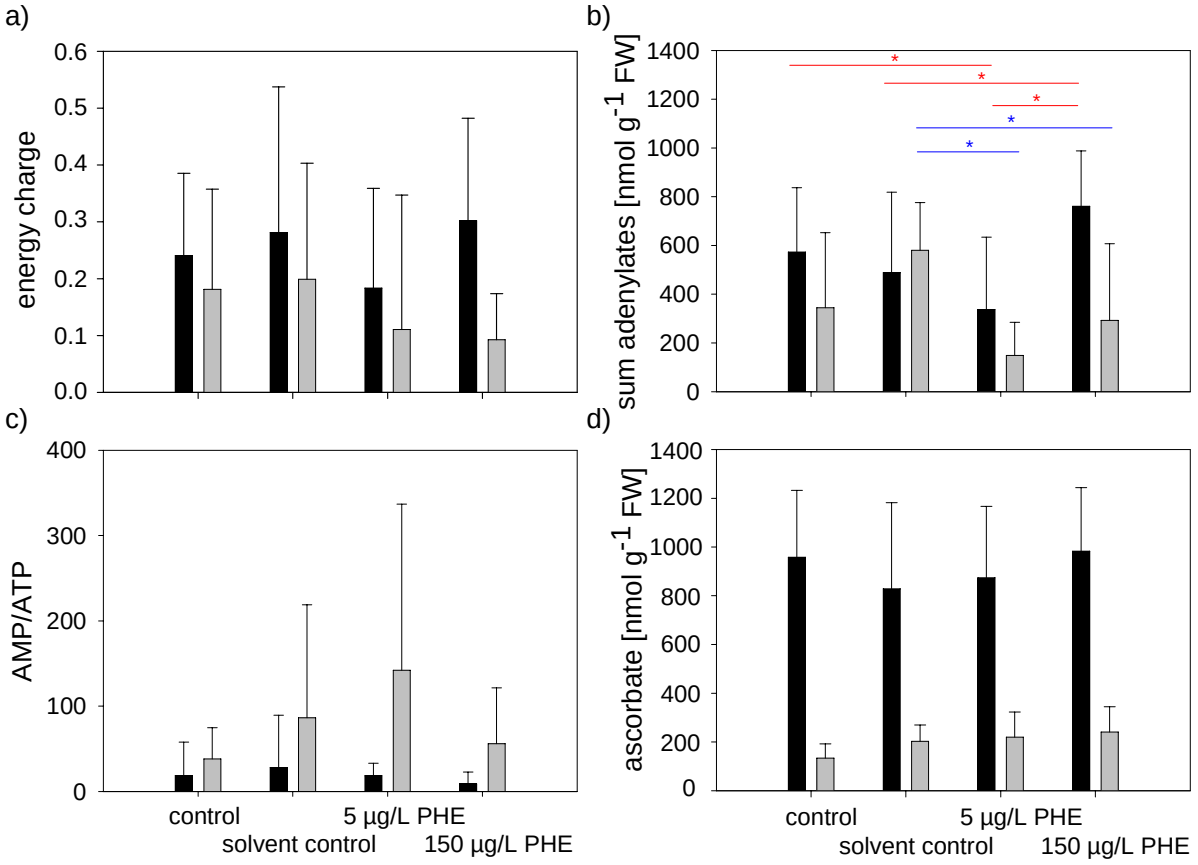

Figure 3: Tissue metabolites in gonads of female (black bars) and male (grey bars) *P. miliaris*. a) Energy charge (2-way ANOVA, sex: p < 0.001, treatment: p = 0.188, interaction, p = 0.600), b) total adenylates in nmol g^{-1} fresh weight (2-way ANOVA, sex: p = 0.002, treatment: p = 0.004, interaction, p = 0.024). Asteriks show significant differences between treatments within females (red) and within males (blue), c) AMP/ATP ratio (2-way ANOVA, sex: p < 0.001, treatment: p = 0.416, interaction: p = 0.839), d) ascorbate in nmol g^{-1} fresh weight (2-way ANOVA, sex: p < 0.001, treatment p = 0.217, interaction: p = 0.070).

Furthermore, treatment and the interaction of treatment x sex had significant effects on total adenylates (2-way ANOVA, sex: p = 0.002, treatment: p = 0.004, interaction, p = 0.024, Fisher LSD, p < 0.05). Within females, significantly higher adenylate values were found in the 150 µg L^{-1} phenanthrene-treated group compared to the solvent control and the 5 µg L^{-1} phenanthrene-treated group. Moreover, controls had a significantly higher total adenylate values than the 5 µg L^{-1} phenanthrene-treated group. Male sea urchin had significantly lower adenylate concentrations in both phenanthrene-treated groups compared to controls. In general, the differences in sum

adenylates between treatments reflect changes of all adenylates (AMP, ATP, ADP) (compare Table 3), and not of a specific component.

The AMP/ATP ratio (Figure 3c) was 80 ± 125 in males, around four times higher than in females. Treatment had no significant effect on the ratio of AMP/ATP (2-way ANOVA, sex < 0.001, treatment: p = 0.416, interaction p = 0.839). The different AMP/ATP ratios in females and males result from significantly higher ATP levels in females, whereas AMP levels did not differ between sexes (Table 3). However, treatment, as well as the interaction between sex and treatment, had significant effects on AMP concentrations (2-way ANOVA, treatment p = 0.016, sex p = 0.228, interaction p = 0.031). Combining data of females and males, AMP levels were significantly lower following exposure to 5 µg L^{-1} phenanthrene compared to the three other groups. Within females, AMP concentrations differed only between the 5 µg L^{-1} vs. the 150 µg L^{-1} phenanthrene group. Moreover, in females AMP levels were significantly elevated in the 150 µg L^{-1} phenanthrene treatment compared to the solvent control. In contrast, AMP levels in males were significantly lower in the 150 µg L^{-1} phenanthrene vs. the solvent control group. In the 150 µg L^{-1} group, AMP values were also significantly lower in males compared to females.

Table 3: Ascorbate and adenylate concentrations in gonads of female (f) and male (m) sea urchins as nmol g^{-1} fresh weight in the different experimental treatments. Different letters indicate signifcant differences between sex and / or treatment (2-way ANOVA: AMP: treatment p = 0.016, sex p = 0.228, interaction p = 0.031; ADP: treatment p = 0.005, sex p < 0.001, interaction p = 0.048; ATP: treatment p = 0.013, sex p< 0.001, interaction p = 0.214). When sex and treatment have an interactive effect on adenylates (in the case of AMP and ADP) differences between treatments are indicated with different letters in red (within females) and in blue (within males) while differences in sexes within treatments are indicated with green symbols. In the case of ATP no interactive effect of treatment and sex were found and different letters (in black) indicate significant differences within females and males.

treatment	sex	n	AMP [nmol g^{-1} FW]	ADP [nmol g^{-1} FW]	ATP [nmol g^{-1} FW]
control	f	11	342 (109) ^{ab}	124 (87) $^{ab\,\alpha}$	107 (104) ^{a}
control	m	9	275 (232) ^{ab}	56 (86) $^{ab\,\beta}$	14 (20) ^{b}
solvent control	f	11	242 (166) ^{a}	100 (87) ^{b}	147 (229) ^{a}
solvent control	m	9	378 (124) ^{a}	117 (107) ^{b}	86 (116) ^{b}
5 µg L^{-1} phenanthrene	f	10	203 (159) ^{ab}	67 (87) ^{a}	66 (97) ^{c}
5 µg L^{-1} phenanthrene	m	9	140 (127) ^{b}	6 (10) ^{a}	3 (3) ^{d}
150 µg L^{-1} phenanthrene	f	8	430 (147) $^{b\,\alpha}$	171 (97) $^{b\alpha}$	160 (125) ^{a}
150 µg L^{-1} phenanthrene	m	9	237 (227) $^{b\,\beta}$	43 (75) $^{b\,\beta}$	12 (17) ^{b}

ATP concentrations differed significantly between treatments and between sexes (2-way ANOVA, treatment $p = 0.013$, sex $p < 0.001$, interaction $p = 0.214$). Levels of ATP were significantly higher in females than males and lower in the 5 µg L^{-1} phenanthrene group than the three other groups. Similarly, concentrations of ADP were higher in females than in males and lower following exposure to 5 µg L^{-1} phenanthrene compared to the other treatments. Sex and treatment had an interactive effect on ADP levels (2-way ANOVA, treatment $p = 0.005$, sex $p < 0.001$, interaction $p = 0.048$). ADP concentrations were significantly lower in males than in females in the control and in the 150 µg L^{-1} groups. Within females, the 5 µg L^{-1} group had significantly lower ADP values than the 150 µg L^{-1} exposure group and the seawater control. Likewise, exposure to 5 µg L^{-1} phenanthrene resulted in significantly lower ADP values than the 150 µg L^{-1} and the solvent control within males.

Ascorbate levels in females were 906 ± 296 nmol g^{-1} fresh weight and five times higher than in males where ascobate ranged at 199 ± 92 nmol g^{-1} fresh weight (Figure 3d). Whereas sexes differed significantly, no effect of treatment on ascorbate levels was detected (2-way ANOVA, treatment: $p = 0.217$, sex < 0.001, interaction $p = 0.070$).

Discussion

In the present study, we could generally observe either no or little effects of phenanthrene in gonads of the sea urchin *Psammechinus miliaris*. However, the data indicate significant sex-specific differences in histological indices, as well as in gonad energetic status and ascorbate levels.

Pronounced accumulation of lipofuscin in fish liver and mussel digestive gland is associated with oxidative stress caused by pollution with oil and PAHs (Krishnakumar et al. 1994; Krishnakumar et al. 1997; Au et al. 1999; Au 2004). In the present study, lipofuscin accumulation, however, is not increased in the gonads of *P. miliaris* after exposure to the PAH phenanthrene. This confirms a previous study where exposure to a higher concentration of phenanthrene (500 µg L^{-1}) for a longer period (20 days) did not result in elevated amounts of lipofuscin in ovaries of *P. miliaris* (Schäfer and Köhler 2009). Since exposure to the heavy metal lead also failed to induce lipofuscin accumulation in sea urchin ovaries (Schäfer and Köhler unpublished data), fluorescent age pigment accumulation does not appear to be an appropriate indicator for pollutant-induced stress in gonads. Instead, formation of lipofuscin in reproductive tissue of sea urchins depends on gonadal stage: In *P. miliaris* sampled at monthly intervals in the northern Wadden Sea, highest

levels of lipofuscin were found at the end of gametogenesis in partially spawned or spent animals (Schäfer and Köhler unpublished data). In keeping with our present study, the field samples revealed significantly higher levels of lipofuscin in males than females (Schäfer and Köhler unpublished data). Fibrosis of the acinal wall is also found significantly more often in partially spawned and spent animals (Schäfer and Köhler unpublished data), and in the present study male *P. miliaris* exhibited more severe fibrosis than females.

PAHs and oils are known to increase the prevalence of oocyte atresia in sea urchins (Schäfer and Köhler 2009) as well as in other invertebrates (e.g. Lowe and Pipe 1986; Tay et al. 2003; Ortiz-Zarragoitia and Cajaraville 2006). In the present study, oocyte atresia was not induced by exposure to 5 or even 150 µg phenanthrene L^{-1} for 10 days, and only exposure to as much as 500 µg L^{-1} phenanthrene for 20 days increased atresia of previtellogenic oocytes in a previous study (Schäfer and Köhler 2009). Oocyte atresia is, however, induced by the untimely release of gametes in the premature females (Figure 2). Possibly, either the concentrations we used here (5 and 150 µg L^{-1}) are too low to cause oocyte atresia within 10 days of exposure or spontaneous spawning events superimpose the pollutant-mediated response. Interestingly, in the above mentioned study, we could not observe signs of degeneration in vitellogenic oocytes and mature eggs, which implies the later developmental stages of oocytes to be better protected against pollutant stress (Schäfer and Köhler 2009). In the present study, atresia, however, was observed in all stages of oocytes as well as in ova. This indicates that degeneration of vitellogenic oocytes and ova result from spawning-induced phagocytosis of relict gametes. Alternatively, different timing of the experiments in regard to the reproductive cycle of the sea urchins may be responsible for the inconsistent observations. In contrast to the previous study (Schäfer and Köhler 2009), we also failed to observe either aggregation of degenerating oocytes, disorganisation of the acinal structure, or changes in nutritive phagocytes after exposure of sea urchins to 5 and 150 µg phenanthrene L^{-1}.

The low EC values of around 0.2 and the high AMP/ATP ratios largely exceeding values of 1 may be surprising at first glance: Typical EC values for optimal conditions are in the range 0.8 to 0.9 while stress conditions yield values in the range of 0.5 to 0.7 or lower (Ivanovici 1980; Verschraegen et al. 1985). Furthermore, ATP values usually exceed AMP concentrations in tissues and cells (Ataullakhanov and Vitvitsky 2002). As demonstrated in the present study, sea urchin gonads are very distinct from other tissues. We suggest that the low EC values and the high AMP/ATP ratio reflect a predominant anaerobic metabolism with minimal oxidative phosphorylation in the gonads. Bookbinder and Shick (1986) demonstrated that up to 92% of the

heat dissipated by isolated ovaries of the sea urchin *Strongylocentrotus droebachiensis* derives from anaerobic metabolism. The ovaries have the capacity to produce large amounts of lactate but other unknown, anaerobic end products are also supposed to be present in sea urchin ovaries (Bookbinder and Shick 1986). It is well known that the key glycolytic enzyme phosphofructokinase is activated by AMP and inhibited by ATP (Ataullakhanov and Vitvitsky 2002). The high AMP/ATP ratio found in the gonads of *P. miliaris* probably upregulates glycolysis to provide low levels of ATP via this pathway. Anaerobiosis in gonadal tissue may be advantageous for the protection of the developing gametes against reactive oxygen species as these are usually formed during oxidative phosphorylation in the electron transport chain inside of the mitochondria (Turrens 2003). Mutagenesis of gametes would otherwise result in non-viable offspring and consequently compromise species survival.

Furthermore, cellular metabolism is predominantly inactive in unfertilised eggs but increases after fertilization (Epel 1978). Next to energy provided by anaerobic metabolism, energy demands of gametes are covered in sea urchins by the delivery of nutrients from the gut. In echinoids, gonads function not only as reproductive organ but also as nutrient store (Walker et al. 1998). Nutrients accumulate in the somatic cells of the gonads during gonadal growth and are either consumed (both sexes) or delivered to the gametes (females) during gametogenesis (Walker et al. 1998; Unuma et al. 2003). A main constituent of yolk in sea urchins is the major yolk protein (MYP), an iron-binding transferrin-like protein. In contrast to vitellogenins, MYP is not female-specific (Brooks and Wessel 2002). The MYP is synthesized in the adult gut, transported to the gonads and, in females, selectively packaged into the yolk platelets of oocytes (Brooks and Wessel 2004).

In contrast to our results, Luk'yanova (1994) measured EC values of around 0.9 in gonads of the sea urchin *Strongylocentrotus intermedius*. Presently, we are unable to explain these differences. Nonetheless, they may result from seasonal differences in the energy metabolism of sea urchin gonads: In the Pacific oyster (*Crassostrea gigas*) the reproductive cycle is known to affect the EC of whole soft tissues due to energy consumption during gametogenesis (Delaporte et al. 2006). And in the mussel *Mytilus edulis* ATP concentrations and EC ratios in the soft tissues could be related to the annual reproductive cycle and are supposed to reflect changing weight-proportions of hepatopancreas and gonads (Skjoldal and Barkati 1982). Furthermore, invertebrates are known to tolerate lower EC values (0.3 -0.4) than vertebrates (0.5-0.6) due to a less stringent regulation and a low efficiency of the enzyme AMP deaminase (Raffin et al. 1994 cited in Marazza et al. 1996).

AMP deaminase regulates cellular energy charge by degradation of AMP resulting in an increase in EC (Ataullakhanov and Vitvitsky 2002).

Tissue metabolites further reflect significant sex-specific differences in energy metabolism of sea urchin gonads: Females exhibit significantly increased EC values, total adenylates as well as ATP levels. Interestingly, Allen (1996) hypothesized that the female germ line possesses undifferentiated mitochondria incapable of ATP production for protection of oocytes from free radicals. This mechanism shall ensure the integrity of mitochondrial DNA and its transfer to the next generation. Mitochondria of the short-lived sperm are, in contrast, supposed to be committed to short-term energy production for maximal motility of sperm during fertilization (Allen 1996). In sea urchin sperm the mitochondrion is located at the base of the sperm head and mitochondrial respiration is activated to fuel motility. For sperm motility ATP generated by oxidative phophorylation must be actively transported down the length of the tail where it is hydrolyzed by dynein ATPase for bending waves along the flagellum. Sea urchin spermatozoa utilize the phophocreatine shuttle, where flagellar creatine kinase uses phosphocreatine to re-phospharylate ADP (Tombes and Shapiro 1985). Furthermore, sperm possess an adenylate kinase (AK) which uses 2 ADP to produce ATP + AMP. Adenylate kinase activity in sperm was first demonstrated by Schoff et al. (1989) in bovine sperm flagella and has recently been investigated in sea urchin sperm (Kinukawa et al. 2007). Kinukawa et al. (2007) estimated that AK contributes to around 30 % and phosphocreatine kinase to 70% of non-mitochondrial ATP synthesis utilized for sperm motility. In murine testis, two adenylate kinases have been identified with AK1 being first expressed in condensing spermatids while AK2 being expressed throughout spermatogenesis (Cao et al. 2006).

A second hint towards better antioxidant protection in female reproductive tissues are significantly higher levels of the antioxidant ascorbate compared to males. In general, testes of different animal phyla are known to contain a high amount of polyunsaturated fatty acids which are prone to oxidation (Halliwell and Gutteridge 2007). In *P. miliaris* significantly increased levels of polyunsaturated acids were found as well as a dramatic reduction in the fatty acids 22:6(n-3) and 22:5(n-3) at increasing maturity (Hughes et al. 2006). Ovaries may, therefore, be less susceptible to oxidative stress than testes, with higher ascorbate levels resulting in an increased ROS scavenging potential, and due to the presence of less oxidizable compounds. The result is a lower lipid and protein peroxidation state and finally less lipofuscin deposition in female sea urchins, as documented here, as well as in the field study (Schäfer & Köhler unpublished). Furthermore, we propose that lower ROS scavenging capacity might be responsible for the higher

incidence of fibrosis in males compared to females, since fibrosis is suggested to be a scarring process in sea urchins (Schäfer & Köhler unpublished), as well as in fish (Dietrich et al. 2009).

The lower concentration (5 µg L^{-1}) of phenanthrene used in the present study was selected as it is around the 'safe' level of phenanthrene for aquatic organisms set by several countries (Law et al. 1997). In addition, it is in the range of background concentrations for total PAH concentrations reported in seawater around England and Wales (Law et al. 1997). After exposure to 5 µg L^{-1} phenanthrene for 10 days we do not observe any behavioural changes (food intake), growth inhibition or histopathological alterations in the gonads of *P. miliaris*. Yet, biochemical analysis documented decreased levels of ATP in sea urchins treated with 5 µg L^{-1} phenanthrene compared to control treatments, whereas levels remained unchanged after treatment with 150 µg L^{-1}. Increased energy consumption following exposure to the lower phenanthrene concentration may be an initial adaptative response to cope with higher energy demand required for cellular detoxification and protection mechanisms. At the higher phenanthrene concentration (150 µg L^{-1}) sea urchins may no longer be capable to control on toxic effects and energy is not spent optimal. Similar responses are proposed by Marazza et al. (1996) for the energy metabolism in shrimp stressed by exposure to ammonia. The reduced food intake of *P. miliaris* observed after exposure to 150 µg L^{-1} further shows that, despite any clear histopathological or biochemical changes occuring in the gonads, behaviour is severely affected.

The complex responses of sea urchins to phenanthrene highlight the need for measuring various parameters at different levels of biological organisation (tissue, individual) for detection of xenobiotically mediated adverse effects. Moreover, this investigation illustrates the difficulty for the identification of cause effect relationships of toxic injury in gonads, as they may be superimposed by the seasonality, as well as by the release of gametes. We suggest that the sex-specific differences of histopathological as well as biochemical parameters in the gonads of *P. miliaris* are related to the specific function of the gonadal tissue and reflect adaptative mechanisms of gametes to different life spans – the long-lived eggs and the short-lived sperm. In this regard, it has to be mentioned that in invertebrates sperm are often reported to be more susceptible to chemical stress than eggs (Au et al. 2001; Caldwell et al. 2004; Fitzpatrick et al. 2008). In the sea urchin *Anthocidaris crassipina*, for example, chronic cadmium exposure of adult organisms resulted in impaired reproduction of males while fertilization capability of eggs was unaffected (Au et al. 2001). Another example is the blue mussel *Mytilus trossulus* with significantly reduced fertilization rates after exposure of sperm to 100 µg L^{-1} copper but not after exposure of eggs

(Fitzpatrick et al. 2008). The short life span of sperm may not necessitate a high antioxidant scavenging potential and protective mechanisms against reactive oxygen species in the gonads which is supported in the present study by low ascorbate levels, higher amounts of lipofuscin and higher incidence of fibrosis in male compared to female gonads. In gonads of both sexes the use of glycolytic or transphosphorylation energetic pathways lower the risk of producing reactive oxygen species, whereas higher EC levels and lower AMP/ATP ratios in female than male gonads indicate functional differences in energetic metabolism of ovaries and testes.

Acknowledgements

The authors thank the Alfred Wegener Institute for Polar and Marine Research within the Helmholtz Association for financial support. We thank Birgit Hussel and the crew of the MS Mya at the Wadden Sea station on Sylt for collecting the sea urchins. We thank Christiane Schäfer from the Zeiss Company for her help with the Image Analysis and Stefanie Meyer for her support in the laboratory.

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Gonadal histopathology of sea urchins: its application in toxicology

Schäfer, S., Köhler, A., *in preparation*.

Abstract

Histopathology of gonadal tissues may be a powerful tool for the assessment and the early detection of xenobiotically mediated adverse effects on reproductive function. For sea urchins, however, only few studies have been published investigating histological changes in gonads after chemical exposure. In addition, the terminology of the diagnostic criteria and the descriptions of the findings are often insufficient. In this paper, general considerations for the application of gonadal histopathology in sea urchins using light microscopy are presented. Experiences and results from our own laboratory studies with the sea urchin *Psammechinus miliaris* are complemented with literature data.

key words

histopathology, gonad, sea urchin, oocyte atresia, sperm necrosis

Gonad staging is essential for histological analysis of reproductive tissues. The reproductive cycle of sea urchins is, in general, divided in five to six stages, namely the recovery, growing, premature, mature, (partially spawned) and spent stage. Though, suitable gametes for the histopathological assessment of the reproductive tissues are only present in the growing, premature and mature stages. In the northern latitudes most sea urchin species have an annual reproductive cycle with one spawning period. Since many sea urchin species are important for fishing or aquaculture detailed descriptions of the gonadal stages, the respective spawning periods and the developmental stages of the germ cells are available.

Sea urchins are dioecious organisms and due to their pentaradial body organisation they possess five gonads with separate gonadopores. Functional units of sea urchin gonads are so-called acini which are surrounded by a multi-layered acinal wall. Gametes are formed at the inner epithelial layer of the acinal wall and migrate towards the center of the acini during maturation. In mature animals the acini are densely packed with mature gametes.

In accordance to studies with other aquatic species, degenerative processes of germ cells, such as oocyte atresia, sperm necrosis and resorption of gametes, are the most common histopathological parameters investigated in sea urchins (Khristoforova et al. 1984, Vaschenko et al. 2001, Vashchenko and Zhadan 1993, Vashchenko et al. 2001, Schäfer and Köhler 2009a). However, direct comparisons of the observed effects are hardly possible as detailed descriptions and adequate micrographs of the findings are often missing. Additionally, the diagnostic criteria used are often not defined. We, therefore, recommend the stringent use of consistent nomenclature and propose basic diagnostic criteria for gonadal histopathology of sea urchins (Table 1) which should be upgraded in future studies.

Table 1: Proposed diagnostic criteria for gonadal histopathology of sea urchins.

accompanying parameters	remarks		
gonad index	• reduced gonad index may indicate tissue atrophy • dependend on the reproductive stage		
sex	• affects several histological parameters		
reproductive stage	• altered reproductive stage may be indicative for abnormal or asynchronous maturation • affects several histological parameters		
spawning	• untimely spawning events may be induced by chemical stress • spawning favours degeneration and resorption of gametes • quantification in tissue samples recommended		
tissue structure or cellular component	diagnostic criteria	characteristics	remarks
acinal structure	disorganisation	• gametes, preferebly early developmental stages, are spread in the acini • usual tissue morphology disturbed	• may result from aggregation of degenerating germ cells
germ cells	oocyte atresia	• fading and vacuolisation of the cytoplasm • infoldings and disruption of the nuclear membrane • finally cell lysis	• needs to be carefully evaluated in animals that had underwent spontanenous spawning events
	aggregation of degenerating oocytes	• aggregates of degenerating oocytes showing primarily atretic characteristics • disturbing tissue morphology	• may result in disorganisation of acinal structure
	sperm necrosis	• shrinking and darkening (pyknosis) of the nucleus • fragmentation of the nucleus (karyorrhexis)	• needs to be carefully evaluated in animals that had underwent spontanenous spawning events
	decreased number of gametes		• may be indicative for altered maturation • may be indicative for spawning events
	proliferation of oogonia or spermatogonia	• increased numbers of oogonia or spermatogonia	
somatic cells	morphological changes		• need to be characterized in detail

Table 1: continued

acinal wall	fibrosis	• dilation of the epithelial layers of the acinal wall • augmentation of collagenous, stronly eosinophilic material between epithelial layers • possibly increased formation of basophilic material between epithelial layers	
others	immune reaction		• immune cells may be present outside of acini especially in conjunction with lipofuscin granules • immune cells may invade acini • subpopulations of immune cells need to be characterized *in situ*

Next to effects on specific cells, gametogenesis itself may be abnormal or asynchronous after chemical exposure (Vashchenko et al. 2001). From studies with other aquatic invertebrates and fish further parameters are known which may be indicative for chemically induced alterations: In fish several alterations of the accessory cells are described such as proliferation and hyperplasia (increase in cell numbers), hypertrophy (enlargement) or vacuolisation (Blazer 2002) which may aid in the identification of analogous changes in sea urchins. In addition, ovotestis or intersex is often found in aquatic organisms after exposure to endocrine disruptors. In many sea urchin species, hermaphrodites have been described but, to our knowledge, their occurrence has so far not been linked to exposure towards endocrine disruptive chemicals.

Different factors may significantly confound histopathological findings. Accumulation of lipofuscin, an end product of protein and lipid peroxidation due to oxidative stress, for example, was observed in gonads of *Strongylocentrotus intermedius* (Vaschenko et al. 2001; Vashchenko et al. 2001). The authors linked the lipofuscin accumulation to heavy metal pollution of the sea urchin habitat. However, reproductive stage and sex may significantly affect lipofuscin content in sea urchin gonads: In *P. miliaris* lipopigment deposition was increased in males compared to females and was highest in partially spawned or spent animals of both sexes (Schäfer and Köhler unpublished). In laboratory studies, lipofuscin accumulation in ovaries of *P. miliaris* could further not be correlated with exposure to the polycyclic aromatic hydrocarbon phenanthrene (Schäfer and Köhler 2009a) or the heavy metal lead (Schäfer and Köhler 2009b). Sea urchins are able to shed part of their gametes before the actual spawning period (Spirlet et al. 1998). Thereby, affected oocytes and sperm may have been released before sampling which may result in underestimating the adverse effects of environmental pollutants on gametes. Next to environmental stressors, spawning events may be induced by handling stress during experiments or sampling. Spawning should be monitored whilst conducting experiments since chemical stress can as well induce the untimely release of gametes (Cajaraville et al. 1992). Moreover, we recommend the quantification of spawning in individual samples since spawning favours the degeneration and resorption of gametes.

Histopathological findings such as abnormal gametes can easily be quantified manually by counting their frequency. Eyepiece graticules such as micrometer scales, squared grids and the Weibel multipurpose system can be applied for manual histomorphometric measurements (e.g. oocyte size, volume density of degenerating germ cells). For routine studies we recommend the

implementation of computer-assisted image analysis for histomorphometric (Wolf et al. 2004) and cytochemical evaluation (Schäfer and Köhler in press) of gonadal tissues.

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Measuring of Ca^{2+}-signalling at fertilization in the sea urchin *Psammechinus miliaris*: alterations of this Ca^{2+}-signal by copper and 2,4,6-tribromophenol

Schäfer, S., Bickmeyer, U., Köhler, A. *published in* Comparative Biochemistry and Physioloy C (2009) 150: 261-269.

Abstract

During fertilization, eggs undergo a temporary rise in the intracellular concentration of free Ca^{2+} ions. Using the membrane permeable acetoxymethylester of the fluorescent calcium indicator dye Fura-2, Fura-2 AM, the Ca^{2+}-signal at fertilization was not detectable in eggs of the sea urchin *Psammechinus miliaris*. However, after treatment of the eggs with Fura-2 AM in combination with MK571, an inhibitor for multidrug resistance associated proteins, clear Ca^{2+}-signals at fertilization could be measured without microinjection of the dye. We used this methodology to detect possible alterations of Ca^{2+}-signalling at fertilization by exposure of eggs to environmental pollutants. For this purpose, the heavy metal copper, the bromophenol 2,4,6-tribromophenol, the organic compound bisphenol A and the polycyclic aromatic hydrocarbon phenanthrene were tested for their potential to inhibit fertilization success of *P. miliaris*. Copper and 2,4,6-tribromophenol showed a dose-dependent effect on fertilization rates of *P. miliaris* and significantly inhibited fertilization at 6.3 µM Cu^{2+} and 1 µM 2,4,6-tribromphenol. Bisphenol A significantly inhibited fertilization success at 438 µM while phenanthrene had no effect up to 56 µM. 6.3 µM copper and 100 µM 2,4,6-tribromophenol significantly increased the Ca^{2+}-signal at fertilization. This alteration may contribute to the reduced fertilization rates of *P. miliaris* after exposure to copper and 2,4,6-tribromophenol.

Key words

calcium signalling, copper, fertilization, MK571, sea urchin, 2,4,6-tribromophenol, MRP

Introduction

Free calcium ions are essential second messengers in cells from their origin at fertilization throughout their entire lifespan (Carafoli 2002). Disruption of cellular Ca^{2+} homeostasis appears to mediate the toxicity of many chemicals (Nicotera et al. 1992). Sustained increase in intracellular Ca^{2+} can provoke cytotoxic mechanisms in various cells and tissues by activation of Ca^{2+}-dependent enzymes, alterations of the cytoskeleton, mitochondrial damage, and by the activation of irreversible catabolic processes which may ultimately result in cell death (Nicotera et al. 1992; Stohs and Bagchi 1995; Nicotera and Orrenius 1998). A diverse range of natural and anthropogenic chemicals such as divalent heavy metal ions, bromophenols, bisphenol A as well as polycyclic aromatic hydrocarbons have been shown to interfere with cellular Ca^{2+}-signalling (e.g. Büsselberg et al. 1990; Davila et al. 1995; Stohs and Bagchi 1995; Nielsen et al. 2003; Wozniak et al. 2005).

At fertilization, eggs undergo an increase in intracellular Ca^{2+} beginning at the point of sperm-egg fusion and crossing the egg to the antipode in a wave-like fashion (Santella et al. 2004; Whitaker 2006). This calcium wave is the first event at fertilization triggering the quiescent egg into metabolic activity by posttranslational activation of enzymes, exocytosis of cortical granules for formation of the fertilization membrane and resumption of the cell cycle (Covian-Nares et al. 2004; Santella et al. 2004).

In the following paragraph a selection of chemicals interfering with cellular Ca^{2+}-signalling and homeostasis are presented: Cu^{2+} is an essential metal ion required for metabolic processes in all eukaryotes but can reach toxic levels in aquatic environments (Bryan and Langston 1992; Stohs and Bagchi 1995; Zorita et al. 2006). Cu^{2+} has been shown to alter Ca^{2+} signals in developing embryos of the macroalgae *Fucus serratus* (Nielsen et al. 2003). Bromophenols are industrially produced flame retardant intermediates and wood preservatives (Howe et al. 2005) which also occur naturally in the marine environment in algae (Whitfield et al. 1999) as well as in fish and invertebrates (Boyle et al. 1992; Fielman et al. 2001). Recently, bromophenols such as 2,4,6-tribromophenol have been shown to disturb cellular Ca^{2+}-signalling in neuroendocrine cells (Hassenklöver et al. 2006). Bisphenol A, an important key monomer in the production of polycarbonate plastics and epoxy resins, and endocrine disruptor, affects Ca^{2+} homeostasis by provoking Ca^{2+} influx via Ca^{2+} channels in mammalian tumor cell lines (Wozniak et al. 2005). Further, in goldfish bisphenol A significantly altered plasma Ca^{2+} levels (Suzuki et al. 2003). Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants contained in petroleum hydrocarbons and formed during combustion of fossil fuels and other products (Latimer

and Zheng 2003). PAHs and its metabolites have been shown to alter Ca^{2+}-associated signalling pathways in immune (Davila et al. 1995) and nonimmune cells (Barhoumi et al. 2006) as well as in isolated membrane vesicles of mammalian skeletal muscles (Pessah et al. 2001).

Sea urchins are widely used to study the cellular events at fertilization (Santella et al. 2004; Whitaker 2006). Furthermore, some natural and anthropogenic chemicals have been tested on Ca^{2+} homeostasis in sea urchin eggs (Walter et al. 1989; Pesando et al. 1991; Pesando et al. 1996; Girard et al. 1997). Thereby, the permeability of the plasma membrane to Ca^{2+} and other ions as well as the accumulation and release of sequestered Ca^{2+} were assessed (Pesando et al. 1991; Pesando et al. 1996; Girard et al. 1997). Walter et al. (1989) investigated the Ca^{2+} content and uptake of Ca^{2+} as well as the role of mitochondrial damage in sea urchin eggs upon exposure to mercury chloride.

In sea urchins and some other organisms, the calcium wave represents a single event which is followed by a few minor rises in the intracellular concentration of Ca^{2+} ions (Stricker 1999). The mechanisms by which the sperm triggers Ca^{2+} release at fertilization are still under debate (Santella et al. 2004). In the most established model the sperm is believed to introduce a sperm factor into the egg promoting the formation of inositol-1,4,5-triphosphate ($InsP_3$) which initiates the activating Ca^{2+} wave (Jaffe et al. 2001; Santella et al. 2004). Studies indicate that in sea urchins there are two further messengers of Ca^{2+}-signalling: nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP ribose (cADPr) giving the fertilization calcium wave a boost and longevity (Steinhardt et al. 1977; Whitaker 2006). Steinhardt et al. (1977) and Schmidt et al. (1982) have shown that the Ca^{2+} is released from intracellular stores, whereby later $InsP_3$ and cADPr were identified for mobilizing Ca^{2+} from the endoplasmic reticulum (reviewed by Galione, 1994, Jaffe et al., 2001). In contrast, NAADP is known to induce Ca^{2+} release from lysosomes (Churchill et al. 2002).

Calcium signals are mostly measured using fluorescent calcium indicator dyes (Whitaker 2006). The ratiometric fluorescent dye Fura-2 has already been used for measuring the calcium wave at fertilization in eggs of the sea urchin *Lytechinus pictus* (Poenie et al. 1985; Swann and Whitaker 1986) as well as in ascidians and mammals (Hyslop et al. 2001; Carroll et al. 2003). In general, the dyes are microinjected into the eggs. Indeed, Fura-2 is also available as membrane permeable acetoxymethylester Fura-2 AM. After crossing the membrane Fura-2 AM is quickly hydrolyzed by intracellular esterases to produce membrane impermeable Fura-2. Previously, the inhibitor for

multidrug resistance associated proteins (MRP) MK571 has been shown to enhance uptake of fluorescent dyes in animal cells (Manzini and Schild 2003; Bickmeyer et al. 2008) as well as in diatoms (Scherer et al. 2008). MRPs are efflux transporters of the ATP Binding Casette (ABC) superfamily actively transporting and sequestering endogenous and exogenous compounds (Holland and Blight 1999; Leslie et al. 2001). In marine invertebrates MRPs have been demonstrated to be expressed in marine bivalve mollusks as well as in sea urchins (Hamdoun et al. 2004; Lüdeking et al. 2005).

The aim of the present study was to test if chemicals may alter the calcium wave at fertilization in sea urchins. For this purpose, the heavy metal Cu^{2+}, the bromophenol 2,4,6-tribromophenol, bisphenol A, and the polycyclic aromatic hydrocarbon phenanthrene were tested for their potential to inhibit fertilization success of the sea urchin *Psammechinus miliaris*. We measured the calcium wave at fertilization using Fura-2 AM and the MRP inhibitor MK571.

Materials and Methods

Animal collection and maintenance

P. miliaris were collected by fishing subtidal populations close to the Island Sylt (Germany) by a beam trawl with the FK Uthörn in April 2008. Sea urchins were transported to the Biological Institute Helgoland where they were kept in running sea water at ambient temperature until use.

Collection and processing of gametes

P. miliaris were induced to spawn by injection of 0.5 ml 0.5 M KCl. Sperm was collected from the aboral pore of each individual using a syringe fitted to a needle and stored 'dry' on ice until use. Female sea urchins were induced to spawn in the same way, but eggs were released directly into artificial sea water. Gametes were collected during the first 20 minutes of spawning. The quality of eggs was assessed microscopically on the basis of uniformity of shape and size.

In vitro fertilization assays

The following chemicals were tested on fertilization success of *P. miliaris*: copper sulfate ($CuSO_4$ x $5H_2O$), 2,4,6-tribromophenol (TBP), bisphenol A and phenanthrene. 1000x concentrated stock solutions were prepared in distilled water (copper sulfate), DMSO (phenanthrene, bisphenol A), or methanol (2,4,6-tribromophenol). Concentration ranges of test substances were selected according to His et al. (1999), King and Riddle (2001) and Fernández & Beiras (2001) for copper,

Hassenklöver & Bickmeyer (2006) and Hassenklöver et al. (2006) for TBP, Roepke et al. (2005) and Kiyomoto et al. (2006) for bisphenol A and Steevens et al. (1999) and Pillai et al. (2003) for phenanthrene. Eggs and sperm were obtained as described above but eggs were released in general purpose medium 2 (GP 2: 360 mM NaCl, 24.8 mM Na_2SO_4, 8.2 mM KCl, 0.74 mM KBr, 0.09 mM $Na_2B_4O_7$ x 10 H_20, 46.7 mM $MgCl_2$ x 6 H_2O, 11.9 $CaCl_2$ x $2H_2O$, 0.08 mM $SrCl_2$ x 6 H_2O, 2.02 mM $NaHCO_3$) (instead of ASW) which had been aerated for 24 hours (USEPA 1993; Caldwell et al. 2002). During fertilization assays, it is essential to keep the concentrations of sperm and eggs constant in the different treatments and replicates. In preliminary experiments (data not shown), *in vitro* fertilization without exposing the sperm was not satisfactory since washing and counting of the eggs took too long and resulted in low egg quality with insufficient fertilization rates in controls. Therefore, eggs as well as sperm – though only for a short period - were exposed to the test substances during fertilization assays. Thus, reduced fertilization success may be the result of toxicity on both types of gametes.

Eggs from three to four females were pooled and sperm from two to three males were pooled (USEPA 1993). Approximately 200 unfertilized eggs of the pooled egg suspension were stocked in 1 ml medium in polystyrene 24 well microplates. Eggs were incubated at 18°C either with the test substances at set concentrations or with the respective controls (distilled water, DMSO, or methanol). Solvents had a final concentration of 0.1% in all wells. Four replicate incubations were run per treatment. After 60 minutes, 10 µl of sperm suspension in GP2 were added to give a final sperm concentration of 2.5 x 10^6 ml^{-1} (Caldwell et al. 2002). Sperm had been allowed to activate in GP2 approximately 10 minutes prior to use. The plates were gently agitated for 30 seconds to increase sperm / egg encounters and incubated for 15 minutes. Fertilization was stopped by adding 100 µL 4% formaldehyde in ASW. The final concentration of formaldehyde is sufficient to stop fertilization (USEPA 1993) which was evident by amotile sperm and the absence of multi-cellular embryos in the wells. Fertilization success was determined using an inverted microscope (Axiovert 25, Zeiss, Germany) and was defined as elevation of the fertilization membrane observed at 100x magnification. 100 eggs per well were counted and the number of unfertilized eggs were recorded.

Fluorometric measurement of intracellular Ca^{2+} levels at fertilization

For measuring Ca^{2+} signals at fertilization, gametes were obtained as described above but for each test eggs from one female and sperm from one male were used (gametes were not pooled). Eggs were incubated with artificial seawater (ASW: 460 mM NaCl, 10.4 mM KCl, 55 mM $MgCl_2$ x 6 H_20, 11 mM $CaCl_2$, 15 mM Hepes-Na; pH 7.5) containing either 10 µM Fura-2 AM or 10 µM Fura-

2 AM with 50 µM MK571 for 60 minutes at room temperature. Eggs were washed three times with ASW. They were fertilized by adding sperm which were activated by dilution of dry sperm in ASW immediately prior to use.

Fluorescence of eggs during *in vitro* fertilization was monitored by an imaging system (Visitron, Puchheim, Germany) with a CCD camera (Coolsnap) mounted on an inverted microscope (Zeiss Axiovert 100). Two to four eggs were measured simultaneously by using the 'region of interest' function of the software (Metafluor, Meta Imaging Series). Fluorescence was obtained through an UV objective (Zeiss NeoFluar 20x). Data were obtained from division of two images, one obtained at 340 nm, the other at 380 nm excitation. Obtained ratio values were not converted to intracellular Ca^{2+} concentrations.

The effects of Cu^{2+} and TBP on Ca^{2+}-signalling in eggs

Gametes were obtained as described for the fluorometric measurements. In contrast to *in vitro* fertilization assays, counting of eggs is not necessary during fluorometric measurement of intracellular Ca^{2+} levels. Therefore, eggs were washed prior to addition of sperm so that only the eggs were exposed to the tested chemicals.

First, eggs were incubated in medium (GP2) with 10 µM Fura-2 AM and 50 µM MK571 for 60 minutes and washed three times with GP2. Test substances (6.3 µM Cu^{2+} or 100 µM TBP) were added and fluorescence was recorded as described above. Next, it was tested whether the test substances lead to alterations of the calcium wave at fertilization of *P. miliaris*. To avoid interference of the MRP inhibitor on the toxicity of the test substances, eggs were treated with the test substances first and incubated with Fura-2 AM and MK571 afterwards. In detail, eggs were incubated with test substances (6.3 µM Cu^{2+} or 100 µM TBP) for 60 minutes, washed three times with GP2, and then incubated with 10 µM Fura-2 AM and 50 µM MK571 for 60 minutes and washed again three times with GP2. Eggs were fertilized with 10 µL sperm suspension (dry sperm diluted 1:50 immediately prior to use) and fluorescence was recorded. Two to four eggs were recorded simultaneously and at least three experiments were run per treatment. Incubation of eggs and tests were performed at 18°C.

Statistics

Statistical tests were run with Sigma Stat 3.0 (SPSS Inc.). Residuals were tested for normality and variance homogeneity. Data for fertilization rates were arc sin square root transformed and a one-way ANOVA with a Dunnett's test as post hoc test was run as described by the USEPA (1993) for

sea urchin fertilization tests. In case the data were not normally distributed a Kruskal-Wallis ANOVA on ranks was used. The Ca^{2+} signals (the Δ $ratio_{max}$ and the time needed to reach maximal changes in the Ca^{2+} signal) were analysed with a one-way ANOVA and a Dunnett's test. The significance level was set at $p < 0.05$. Note that in Sigma Stat 3.0 the p-values for the Dunnett's test are unavailable. The software only indicates if the p-value is above or below the significance level of 0.05.

Results

In vitro fertilization assays

In figure 1, the fertilization rates of *Psammechinus miliaris* after exposure to the test substances are presented. Exposure to Cu^{2+} significantly reduced fertilization success of *P. miliaris* at the two highest concentrations (6.3 and 63 µM, one-way ANOVA, $p < 0.001$). 63 µM Cu^{2+} significantly decreased the fertilization rate by 76% in comparison to controls. TBP significantly inhibited fertilization success of *P. miliaris* at 1 µM (one-way ANOVA, $p < 0.001$). Exposure to 1000 µM TBP, the highest concentration tested, significantly inhibited the fertilization rate by 75% in comparison to controls. 438 µM bisphenol A significantly reduced fertilization success by 97% in comparison to controls. (one-way ANOVA, $p < 0.001$). Indeed, for bisphenol A no effects were found at concentrations lower than 438 µM. Phenanthrene showed no effect on fertilization success at concentrations of up to 56 µM (Kruskal Wallis ANOVA on ranks, $p = 0.074$).

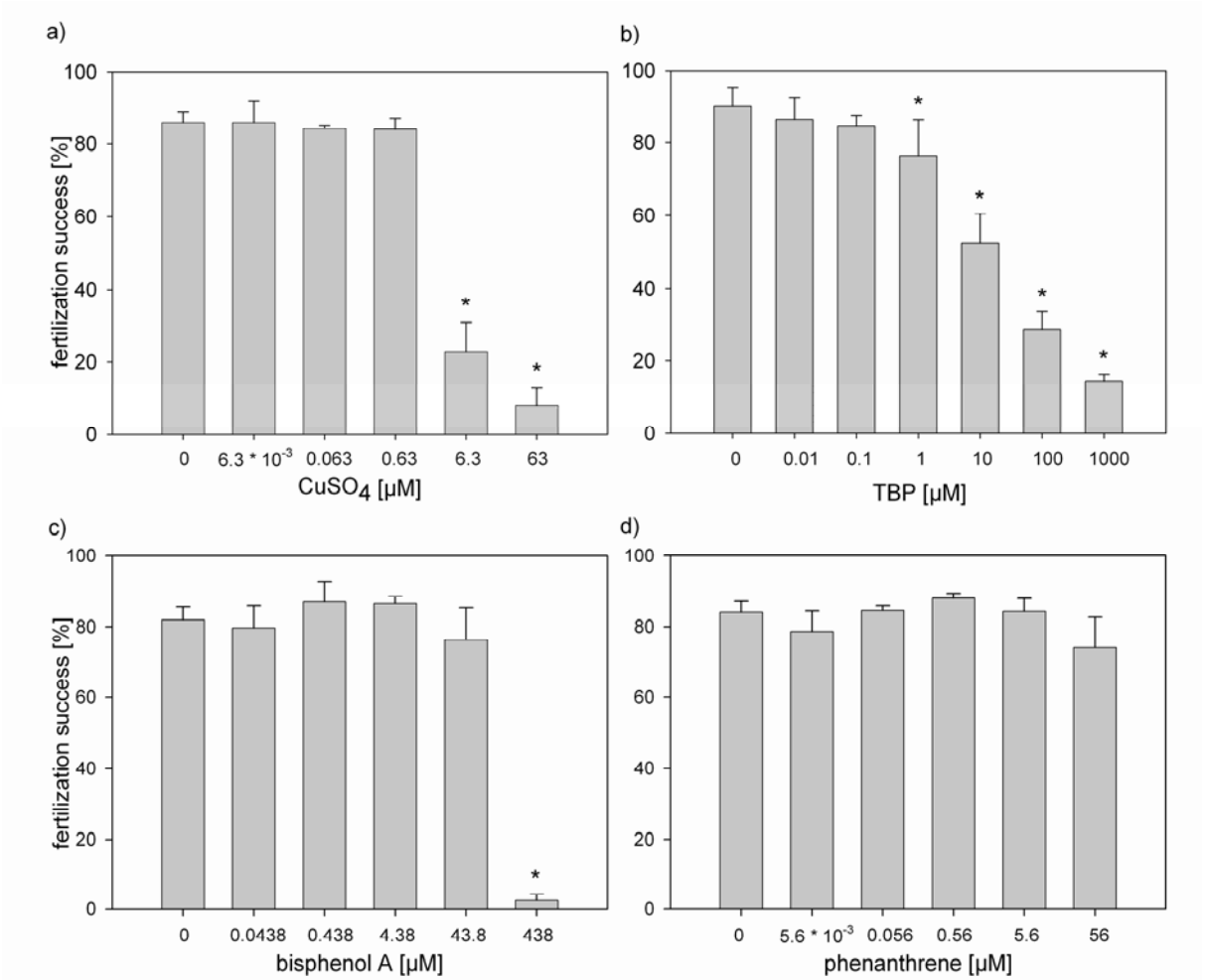

Figure 1: Fertilization success of *Psammechinus miliaris* during exposure to set concentrations [µM] of a) copper sulfate, b) 2,4,6-tribromophenol (TBP), c) bisphenol A and d) phenanthrene. Asterisks indicate significant differences in comparison to controls ($CuSO_4$: one-way ANOVA $p < 0.001$, Dunnett's test $p < 0.05$; TBP: one-way ANOVA, $p < 0.001$, Dunnett's test $p < 0.05$; bisphenol A: one-way ANOVA $p < 0.001$, Dunnett's test $p < 0.05$; phenanthrene: Kruskal-Wallis ANOVA on ranks $p = 0.074$).

Fluorometric measurement of intracellular Ca^{2+} levels at fertilization

In the experiments, eggs of *Psammechinus miliaris* were successfully fertilized by adding activated sperm, which was checked visually after each experiment by elevation of the fertilization membrane. However, after incubation with Fura-2 AM no calcium signals could be observed in the eggs during fertilization (N = 8 eggs measured in three separate experiments). In some cases, an increase in the ratio F340 / F380 was recorded. Indeed, close inspection of the corresponding fluorescence intensities at 340 and 380nm excitation shows that the ratio changes are due to decreasing intensities at both wavelengths induced by cell movement during fertilization (see Fig. 2 a). These changes can not be regarded as alterations in intracellular Ca^{2+} since the fluorescence

spectrum of Fura-2 does not shift in opposite directions with an increase at 340 and a decrease at 380 nm.

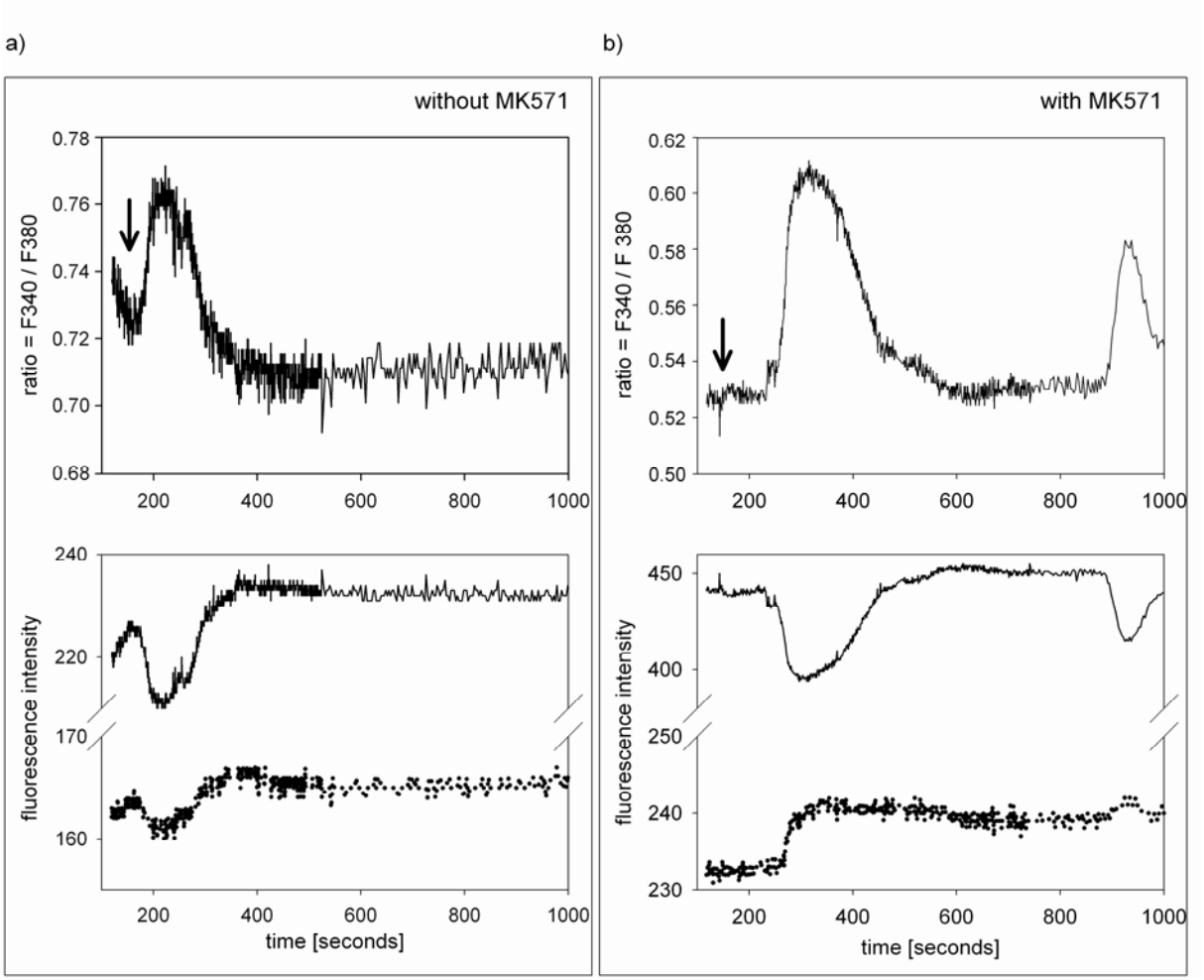

Figure 2: Changes of the ratio F340 / F380 (upper panel) and corresponding fluorescence intensities at 340 nm (dotted line) and 380 nm (solid line) excitation (lower panel) in single eggs of *Psammechinus miliaris* at fertilization. Eggs were incubated with a) 10 µM Fura-2 only and b) 10 µM Fura-2 and 50 µM MK571. Arrows indicate point of adding sperm. Note a second Ca^{2+} rise approximately 400 seconds after the first peak in b).

In contrast, after incubation with Fura-2 AM and MK571 clear calcium waves could be observed in the eggs upon fertilization (Fig. 3) (N = 8 eggs measured in 4 separate experiments). The changes in the ratio of F340 / F380 clearly correspond to changes in intracellular Ca^{2+} since the fluorescence intensity increases at 340 nm and decreases at 380 nm excitation (Fig. 2 b). The ratio of F 340 / 380 changed by 0.033 ± 0.02 in comparison to the resting ratio level before fertilization (N = 8). The calcium waves reached their maximum after 95 ± 33 seconds and lasted 351 ± 130 seconds (N = 8).

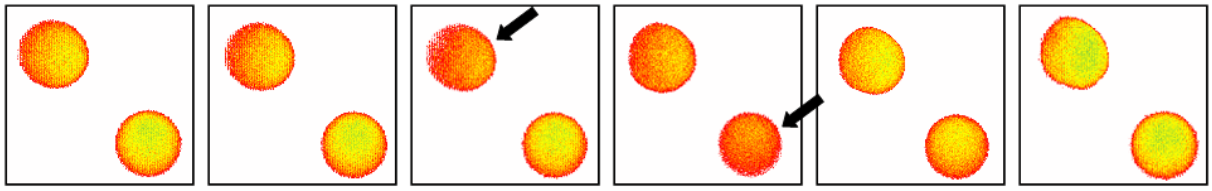

Figure 3: The ratio of F340 / F380 which represent changes of intracellular Ca^{2+} in two eggs of *Psammechinus miliaris* during fertilization (from left to right). Note changes in the ratio F340 / F380 with maximal ratio changes indicated by arrows. Shown are pseudocolored relative fluorescence images of eggs incubated with 10 µM Fura-2 and 50 µM MK571.

In one egg, a second Ca^{2+} rise was observed 10 minutes after the first peak (Fig. 2 b) which was around 70% smaller and with 177 seconds shorter than the first peak with 371 seconds.

The effects of Cu^{2+} and TBP on Ca^{2+}-signalling in eggs

For the following experiments 6.3 µM copper sulfate and 100 µM TBP were selected which inhibited fertilization success by 65% and 61%, respectively, in comparison to controls. Neither 6.3 µM Cu^{2+} nor 100 µM TBP induced detectable calcium signals in unfertilized eggs (data not shown). After incubation of eggs with Cu^{2+} and TBP the fertilizing sperm still induced a Ca^{2+} wave. In figure 4, mean relative changes in the ratio of F340 / F380 at fertilization Δ ratio, obtained after subtracting the resting ratio of F340 / F380 prior to fertilization are shown in the different treatments. Eggs treated with Cu^{2+} and TBP exhibited alterations of the fertilization calcium wave: The maximal changes in the ratio F340 / F380 (the Ca^{2+} peak) were significantly higher in Cu^{2+}- and TBP-treated eggs in comparison to controls (Fig. 5a). In TBP-treated eggs the Δ ratio remained above the resting ratio prior to fertilization within 600 seconds after fertilization, whereas in control and Cu^{2+}-treated eggs the Δ ratio fell to resting levels approximately 400 seconds after fertilization. In addition, the Δ ratio showed a high variance in Cu^{2+}-treated eggs after the fertilization Ca^{2+} wave. Indeed, the time needed to reach the maximal changes in the ratio was not different in the treatments (Fig. 5b).

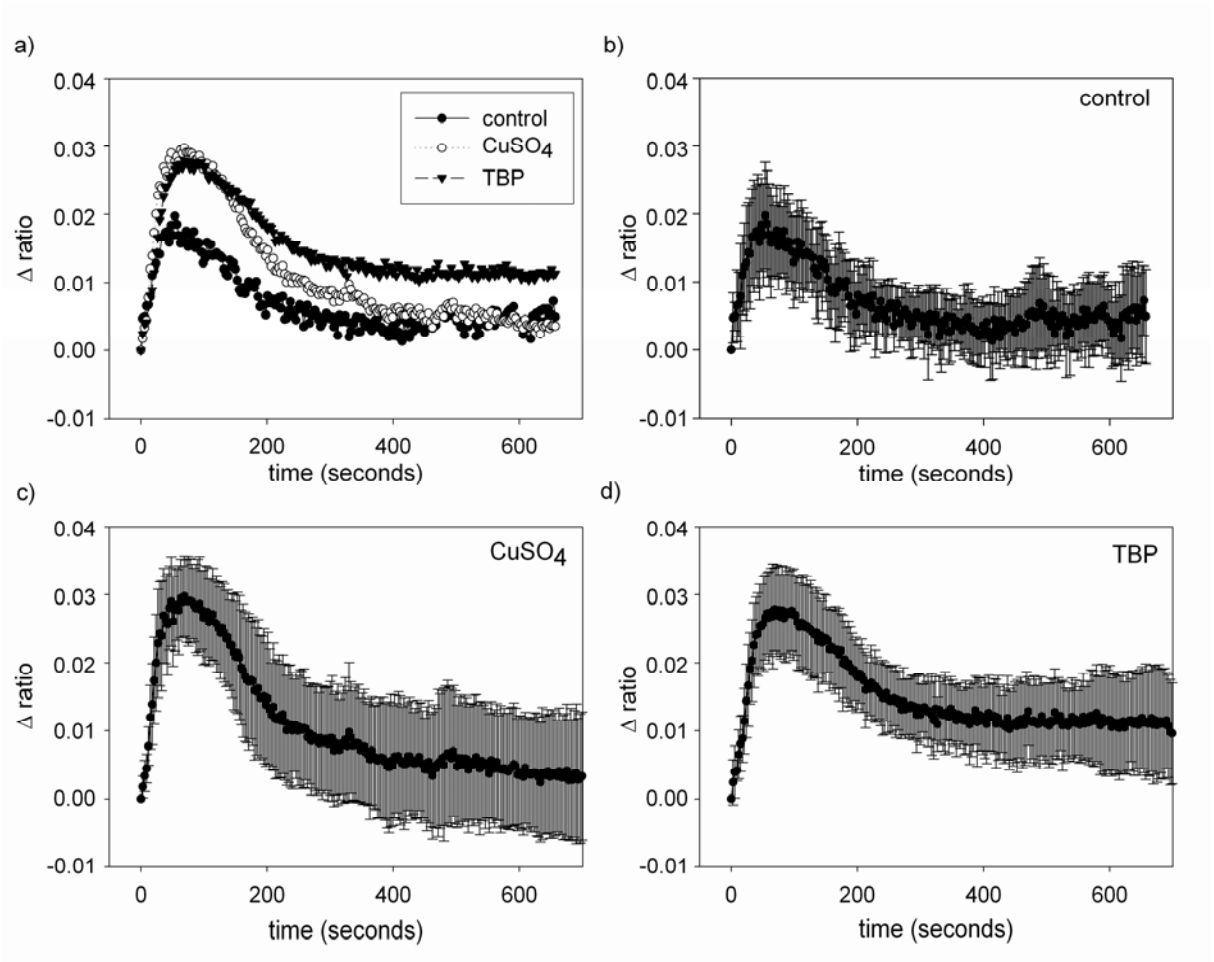

Figure 4: Mean changes in the ratio of F340 / F380 at fertilization in control eggs (N = 8 eggs) and in eggs incubated with 6.3 µM $CuSO_4$ (N = 9) and 100 µM TBP (N = 9), respectively. To obtain Δ ratio values data were subtracted with the respective ratio of F340 / F380 for each egg at the beginning of the fertilization wave. a) Mean Δ ratio for the three treatments, b), c), d) mean Δ ratio values with standard deviations for controls, $CuSO_4$- and TBP-treated eggs, respectively. Data are obtained from three separate experiments per treatment with 2 to 4 eggs each.

In eggs of all treatments, small postfertilization Ca^{2+} rises could be observed within 12 minutes postfertilization (control: 15 postfertilization waves, Cu^{2+}: 5, TBP: 12). In controls up to four and in Cu^{2+}- and TBP-treated eggs up to two postfertilization Ca^{2+} waves were recorded, respectively.

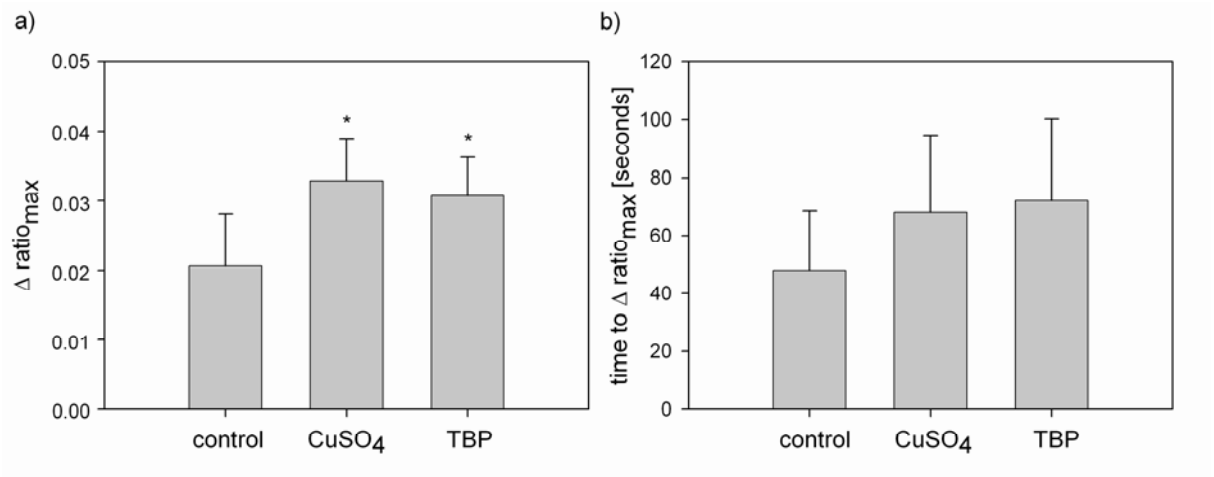

Figure 5: a) Maximal changes in the ratio F340 / F380 at fertilization in control (N = 8 eggs), $CuSO_4$- (N = 9) and TBP-treated (N = 9) eggs. Asterisks indicate significant differences in comparison to controls (one-way ANOVA, $p < 0.001$, Dunnett's test $p < 0.05$). b) The time [seconds] needed to reach maximal changes in the ratio F340 / F380 at fertilization in control, $CuSO_4$– and TBP-treated eggs. No significant differences were found in comparison to controls (one-way ANOVA, $p = 0.143$). Data are obtained from three separate experiments per treatment with 2 to 4 eggs each.

Discussion

So far, studies on the fertilization Ca^{2+} wave have focused on the North American sea urchin species *Lytechinus pictus* and *Strongylocentrotus purpuratus*. Though, Genazzani et al. (1999) have demonstrated that egg homogenates of *P. miliaris* share the same Ca^{2+} release mechanisms as *L. pictus* and *S. purpuratus* with the $InsP_3$, the cADRP and the NAADP pathways. In *L. pictus,* the Ca^{2+} wave at fertilization is reported to peak after approximately 20 seconds (Poenie et al. 1985, Swann and Whitaker 1986), while in the present study, the Ca^{2+} rise in *P. miliaris* takes approximately 95 seconds to reach its maximum. However, the duration of the Ca^{2+} transients are comparable with approximately 5 minutes in *L. pictus* measured by Poenie et al. (1985) and in *P. miliaris* in the present study.

Different developmental stages of marine invertebrates are known to be affected differently by chemicals or polluted water samples with fertilization being either more or less sensitive than embryonic and larval development (e.g. Kobayashi 1980, 1990, Gopalakrishnan et al. 2008). By comparing toxicity of chemicals on different life stages exposure times towards test substances often differ and make a direct comparison difficult. In the present study, fertilization success of *P. miliaris* was inhibited by bisphenol A only at the highest concentration (438 µM) tested. Phenanthrene did not affect fertilization rates of *P. miliaris* up to 56 µM. Yet, these values are

above critical concentrations reported to affect sea urchin embryonic and larval development (Table 1). Furthermore, the effective concentration of phenanthrene is above its 'safe' level of ≤ 29 nM developed by several countries for aquatic organisms (Law et al. 1997). To our knowledge, no maximum permissible value has been set for bisphenol A in the aquatic environment.

We show that fertilization success of *P. miliaris* was significantly affected at 6.3 µM Cu^{2+}. The same concentration increased the calcium wave at fertilization indicating disturbance of Ca^{2+} homeostasis in the eggs by exposure to Cu^{2+}. Early life history stages of marine invertebrates may be particularly sensitive to elevated copper concentrations (Kobayashi 1980; Kobayashi 1990; Bielmyer et al. 2005, Table 1). Generally, divalent heavy metals are known to affect cellular calcium homeostasis (Stohs and Bagchi 1995). Exposure of sea urchin eggs towards the heavy metal mercury chloride increased calcium influx and calcium content in a time and dose-dependent manner resulting in disturbance of mitochondrial function and finally cell death (Walter et al. 1989). In sperm of the mussel *Mytilus edulis* treated with 3.3 mM Cu^{2+}, Ca^{2+} levels significantly decreased in mitochondria and acrosomes indicating an increase in the ionic permability of organelle membranes and possibly resulting in an increase in cytosolic Ca^{2+} (Earnshaw et al. 1986). Ca^{2+} channels on the sperm plasma membrane have also been proposed to be affected after paternal exposure of sea urchins to the heavy metal Cd^{2+} (Au et al. 2001). Furthermore, Cu^{2+} has been shown to alter Ca^{2+} signals in developing embryos of the macroalgae *Fucus serratus*: Moderate Cu^{2+} concentrations (422 nM) inhibited generation of cytosolic Ca^{2+} signals in response to hypoosmotic shock whereas high Cu^{2+} concentrations (2.11 – 8.44 µM) elevated cytosolic Ca^{2+} (Nielsen et al. 2003).

Table 1: Toxicities of test substances on different species of sea urchins (Echinoidea). Presented are the tested chemicals, the tested species, the effective concentration, the duration of the exposure, the tested life stage(s), the observed effect, as well as the reference. n.st. = not stated.

test substance	species	effective concentration	duration of exposure	developmental stage	effect	reference
bisphenol A	*Strongylocentrotus purpuratus*	1 µM	96 hours	embryos-larvae	EC50 for normal development	Roepke et al. 2005
	Hemicentrotus pulcherrimus	≥ 10 µM	directly after till up to 48 hours postfertilization	embryos-larvae	suppression of development	Kiyomoto et al. 2006
	Hemicentrotus pulcherrimus	≥ 10 µM	12 hours after fertilization	embryos-larvae	no effect on development	Kiyomoto et al. 2006
copper	*Heliocidaris erythrogramma*	≥ 4 nM		sperm-eggs	reduced fertilization success, (most sensitive among five tested sea urchin species)	Kobayashi 1980
	Diadema antillarum	44 nM	40 hours	embryos-larvae	EC50 for abnormal development	Bielmeyer et al. 2005
	Paracentrotus lividus	≥ 0.25 µM	48 hours	embryos-larvae	inhibiton of growth	Fernández & Beiras 2001
	Paracentrotus lividus	1.1 µM	48 hours	embryos - larvae	EC50 for complete development	Fernández & Beiras 2001
phenanthrene	*Lytechinus variegatus*	≥ 6 nM	two hours	eggs-embryos	inhibition of embryo development	Steevens et al. 1999
	Lytechinus anemensis	≥ 1 µM	n.st.	embryos	disruption of axial development	Pillai et al. 2003
2,4,6-tribromophenol	*Strongylocentrotus nudus*	≥ 3 nM	one hour	larvae	inhibition of metamorphosis	Agatsuma et al. 2008
	Strongylocentrotus nudus	≥ 30 nM	24 hours	larvae	inhibition of swimming activity	Agatsuma et al. 2008
	Strongylocentrotus nudus	150 nM	24 hours	larvae	mortality	Agatsuma et al. 2008

Next to Cu^{2+}, TBP significantly decreased fertilization success of *P. miliaris* in a dose-dependent manner in our study. Recently, bromophenols have been shown to inhibit larval survival and metamorphosis of the sea urchin *Strongylocentrotus nudus* (Agatsuma et al. 2008, Table 1). In the present study, TBP significantly decreased fertilization success of *P. miliaris* at 10 µM. Moreover, exposure of eggs to 100 µM TBP significantly increased the Ca^{2+} wave at fertilization indicating alterations of calcium signalling by TBP. An increase in intracellular Ca^{2+} partly by Ca^{2+} release from intracellular stores after exposure to TBP has already been shown in neuroendocrine (PC12) cells (Hassenklöver et al. 2006). In a subsequent study, Hassenklöver & Bickmeyer (2006) showed that TBP selectively reduced calcium channel currents in PC12 cells with a half-maximal concentration of 28 ± 18 µM. Additionally, this effect increased with ongoing exposure time (Hassenklöver & Bickmeyer 2006). We, therefore, may strongly underestimate the efficacy of TBP.

In sea water samples from the German Bight up to 6 ng L^{-1} TBP (equivalent to 0.02 nM) are reported while the highest level of all identified bromophenols was 74 ng L^{-1} (Reineke et al. 2006). Higher levels of bromophenols are found in marine sediments and macroalgae: In estuarine sediments from the Rhone river up to 3.7 mg kg^{-1} TBP were found and in the marine macroalgae *Ulva lactua* up to 1.6 mg kg^{-1} TBP have been detected (Howe et al. 2005). Regarding the environmental levels of Cu^{2+}, concentrations range from 0.2 to 2.6 µg L^{-1} dissolved Cu^{2+} (equivalent to 3 to 41 nM) in the North Sea. Indeed, at point sources up to 600 µg L^{-1} dissolved Cu^{2+} (equivalent to 9 µM) can be found (Bryan and Langston 1992). The effective concentrations of Cu^{2+} and TBP tested in this study are, therefore, higher than relevant aqueous concentrations in the marine environment, except for Cu^{2+} at point sources. Regarding TBP, exposure and uptake by sea urchins need to be further investigated in regard to high TBP contamination of sediments in their habitats and the high TBP levels of macroalgae which may serve as food source as already proposed by Agatsuma et al. (2008). In addition, the effective concentrations of Cu^{2+} are above the European safe level of 41 nM Cu^{2+} in marine waters (European Copper Institute 2008). Indeed, it should be considered that during measuring the Ca^{2+} signal at fertilization eggs were not exposed to the tested chemicals to avoid interference of the MRP inhibitor with the test substances. Effective concentrations of Cu^{2+} and TBP on Ca^{2+}-signalling at fertilization in *P. miliaris* are, therefore, likely to be lower than the tested concentrations of 6.3 µM Cu^{2+} and 100 µM TBP, respectively.

In contrast to the effects on the Ca^{2+} wave at fertilization, we were not able to detect induction of Ca^{2+} signals by exposure of unfertilized eggs to Cu^{2+} and TBP. Indeed, the low dye loading of the eggs in comparison to eggs microinjected with indicator dyes in conjunction with the large size of the eggs limits detectability and resolution of intracellular Ca^{2+} changes in these cell types.

We demonstrate that Cu^{2+} and TBP affect calcium signalling at fertilization in sea urchin eggs. This may contribute to the reduced fertilization success of *P. miliaris* exposed to Cu^{2+} and TBP, respectively. Disturbance of Ca^{2+} channels and homeostasis by heavy metals have previously been suggested to affect acrosome reaction and motility of invertebrate sperm (Earnshaw et al. 1986, Au et al. 2001). Since sperm motility is directly correlated with fertilization success (Au et al. 2002) further studies are needed to investigate the effects of Cu^{2+} and TBP on sperm motility of sea urchins. Next to fertilization, early development is regulated by Ca^{2+} (Whitaker 2006) and may be prone to disturbance of calcium homeostasis by TBP and Cu^{2+}. Increased Ca^{2+} levels can trigger the release of hormones from secretory granules but they can also initiate signalling cascades by activation of kinases which may rapidly affect embryonic development. Sustained increase in intracellular Ca^{2+} may further activate Ca^{2+}-dependent degradative enzymes, compromise mitochondrial function and cytoskeletal organization, and ultimately result in cell death.

In the present study, we successfully visualized the calcium wave at fertilization in eggs of sea urchins without using methods risking injury of the cell membrane: In eggs of *P. miliaris* incubated with Fura-2 AM and the MRP inhibitor MK571 we observed clear Ca^{2+} signals at fertilization. Still, it has to be considered that transport inhibitors may reduce fertilization-evoked Ca^{2+}-signals in sea urchin eggs (Davis et al. 2008). In previous studies, Fura-2 was microinjected into eggs of the sea urchin *Lytechinus pictus* to measure the Ca^{2+} rise at fertilization (Poenie et al. 1985; Swann and Whitaker 1986). Simple incubation of eggs with Fura-2 AM has already been performed to investigate Ca^{2+} signals in mouse eggs (Hyslop et al. 2001). However, we could not observe Ca^{2+} signals in sea urchin eggs incubated with Fura-2 AM only. Similarly, Stricker et al. (1992) mentions that in eggs of the sea urchin *Lytechinus pictus* incubated with acetoxymethylesters of the calcium indicator dyes fluo-3 and calcium green no Ca^{2+} wave at fertilization could be detected.

MK571 is a specific blocker for MRP transporters and its application has been suggested to facilitate loading of animals cells (Manzini and Schild 2003; Bickmeyer et al. 2008) as well as diatoms (Scherer et al. 2008) with calcium indicator dyes. Eggs and embryos of the sea urchin

Strongylocentrotus purpuratus are known to express efflux transport activity (Hamdoun et al. 2004). Hamdoun et al. (2004) have shown that the efflux activity is relatively low in unfertilized eggs but is dramatically upregulated within 25 minutes postfertilization possibly by translocation of transporters in vesicles to the plasma membrane (Hamdoun et al. 2004). In sea stars, immunocytochemistry revealed that MRP-like proteins are localized throughout the cytoplasm in oocytes and translocated to the periphery during oocyte maturation (Roepke et al. 2006). After fertilization, eggs of the sea urchin *Lytechinus pictus* microinjected with the calcium indicator fluo-3 show higher fluorescence intensities in the cortex than in the centre of the cell (Stricker et al. 1992). Possibly, in the absence of MK571 Fura-2 is extruded from the cytoplasmic spaces just underneath the plasma membrane by MRP transporters. Changes in intracellular Ca^{2+} which may primarily occur in these cellular regions may, therefore, not be detected when MRP transporters are active.

As demonstrated in the present study, decreasing fluorescence intensities of Fura-2 at 340 and 380 nm excitation induced by cell movements may result in an increasing ratio of F340 / F380. This rise in the ratio of F340 / F380 may be misinterpreted as an increase in intracellular Ca^{2+} concentration. Indeed, Fura-2 changes its fluorescence spectrum upon binding of Ca^{2+} ions in opposite ways: at 340 nm the intensity increases whereas at 380 nm it decreases (Grynkiewicz et al. 1985). The observed decrease in the fluorescence intensity of Fura-2 is due to gross movement of the eggs: Movement of cells is known as major artefact in imaging techniques using fluorescent dyes (Tsien et al. 1985; Silver et al. 1992). In the present study, attacking sperm caused slight movement of the eggs during *in vitro* fertilization. Moreover, eggs changed their shape upon fusion with sperm which is also known from other microscopic studies (Schatten 1981; Stricker et al. 1992).

Acknowledgements

This study was financed by the Alfred Wegener Institute for Polar and Marine Research within the Helmholtz Association. It is part of a PhD study conducted at the Jacobs University Bremen. The authors would like to thank B. Hussel and the staff of the FS Mya for collecting and B. Krüss and A. Röw for maintenance of the sea urchins. We sincerely acknowledge the support of the staff at the Biologische Station Helgoland during the experiments. We would like to thank three anonymous reviewers for their helpful comments on the manuscript.

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Synthesis and perspectives

The presence of various hazardous substances in the environment and related effects on reproductive health of organisms require the assessment and evaluation of potential impacts. Sea urchins were used as model organism for studying pollutant-mediated responses on reproductive function as they are not only popular models in toxicology (Sugni et al. 2007) but their fertilization and embryonic development is also well understood (e.g. Epel 1978; Santella et al. 2004; Whitaker 2006). The outcome of this PhD study is structured in three main topics - gonadal histopathology, sex-specific susceptibility as well as calcium signalling - which will be discussed in detail below.

Gonadal histopathology

Gonadal histopathology is widely used in toxicological studies for detecting the adverse effects of environmental pollutants on aquatic organisms. During the past years, histopathological evaluation of reproductive tissue has gained particular interest for assessing the effects of endocrine disrupting compounds especially in fish (e.g. Dietrich et al. 2009) whereas studies on marine invertebrates and especially on sea urchins are rare and incomprehensive (Khristoforova et al. 1984; Vashchenko and Zhadan 1993; Au et al. 2001; Vaschenko et al. 2001; Vashchenko et al. 2001). In the present study, potential targets for toxic action in gonadal tissue have been identified and described in detail using light microscopy (Chapter IV). Phenanthrene has been shown to affect general gonadal structure, induce atresia of early oocyte stages and result in fibrosis of the acinal wall. The descriptions provide an important basis for future studies on histopathological alterations of reproductive tissues not only in sea urchins but also in other invertebrates. Though, for improving diagnosis of pollutant effects on sea urchin gonads of other classes of chemicals need to be investigated and compared with our previous findings. Another experiment investigating the effects of maternal exposure of *P. miliaris* to the heavy metal lead is under way and will deliver further insights towards this direction. Furthermore, electron microcopic investigations of reproductive tissues in combination with light microscopy may also aid a more detailed understanding of the toxic effects of chemicals for example on oocyte lysosomes (Chapter V; e.g. Nott et al. 1985, Pipe and Moore 1986, Einsporn and Köhler 2008) and will be necessary to investigate adverse effects of chemicals on spermatogenesis (discussed in Chapter VII; Au et al. 2001a, Au et al. 2003).

The complex responses of sea urchins to phenanthrene exposure (Chapter VI) further highlight the necessity to measure parameters at different levels of biological organisation (tissue, individual) to detect xenobiotic-mediated adverse effects. Thus, in the scope of biomonitoring studies using sea urchins additional tissues than the gonads should be analysed as well. Interestingly, Cunha et al. (2005) investigated the potential of glutathione S-transferases (GST) and cholinesterase (ChE) activities of the sea urchin *Paracentrotus lividus* as biomarkers of environmental contamination and identified the ambulacral podia and the anterior part of the intestine as most suitable tissues for such measurements. The collection of ambulacra, being non-destructive, are of great advantage since they allow repeated measurements of one individual or release of specimen alive (Cunha et al. 2005).

For future studies, it would also be desirable to investigate pollutant responses of sea urchin gonads over a complete reproductive cycle as Wintermyer and Cooper (2007) did with the eastern oyster (*Crassostrea virginica*). After initiation of gametogenesis by thermal stress the effects of dioxin treatment over a period of 28 days coinciding with completion of gonadal development were observed (Wintermyer and Cooper 2007). The detailed knowledge about the reproductive biology of sea urchins (e.g. Walker et al. 1998) as well as experiences from commercial sea urchin aquaculture (e.g. Lawrence 2001) will help to develop a similar gametogenesis protocol for sea urchins. Cultivated echinoids have also recently been proposed for marine toxicity tests due to their low biological variation in comparison to field-collected animals (Schipper et al. 2008). Though, it needs to be considered that xenobiotic-mediated responses of animals cultured under optimal conditions and facing – except of the chemical treatment - no other adverse environmental conditions may respond differently to natural populations.

The use of sea urchin gonadal histopathology in ecotoxicology has already been summarized (Chapter VII). Nonetheless, histopathology of reproductive tissues will remain a difficult task as gonads are highly dynamic tissues which have the potential to undergo dramatic and fast changes. Furthermore, we have shown that other factors such as gonadal stage, sex and spawning events confound histopathological findings (Chapters III, VI) and may - if neglected – lead to false interpretations and conclusions. Therefore, experienced and specialized pathologists, critically evaluating histological findings, are needed.

Different susceptibilities of ovaries and testes

Combining histological and biochemical techniques, it was shown that testes are more susceptible to oxidative stress than ovaries: Lipofuscin accumulation and fibrosis were more intense and levels of the non-enzymatic antioxidant ascorbate were significantly lower in male than in female gonads (Chapter VI). Furthermore, energetic parameters differed with testes having significantly lower energy charge values but significantly higher AMP/ATP ratios compared to ovaries. Sex-specific differences in histological and biochemical parameters in the reproductive tissues are suggested to be related to the different life span of female and male gametes as well as functional differences of their mitochondria (Chapter V). Sperms are relatively short-lived, highly specialized cells whose function is to transfer the genetic material of the male germ line. Eggs, on the other hand, live longer, insofar, as they become a zygote and consequently a new organism upon fertilization. Interestingly, the higher susceptibility of male compared to female reproductive tissue is in contrast to studies on other tissues: Higher incidence of liver cancer in female flounder (*Platichthys flesus* L.) has been associated with sex-specific differences in NADPH metabolism involved in xenobiotic biotransformation (Köhler and Van Noorden 2003).

Studies on oxidative stress parameters in sea urchin gonads, such as mitochondrial respiration, activites of antioxidant enzymes (catalase, superoxide dismutase), non-enzymatic antioxidants (glutathione, GSSG:GSH ratio) as well as further parameters of cellular damage (malondialdehyde, protein carbonyls) (Abele et al. 2009), would shed more light on the potential significance of the different life spans of eggs and sperm on the histological and biochemical differences of ovaries and testes found in the present study. Such future studies may also explain the higher susceptibility of invertebrate sperm in comparison to eggs towards pollutants (Au et al. 2001b, Fitzpatrick et al. 2008).

Ca^{2+}-signalling

For the first time, the Ca^{2+} signal at fertilization in sea urchins was demonstrated using the calcium indicator dye Fura-2 AM in combination with the inhibitor for multidrug resistance associated proteins (MRP) MK571. This relatively simple methodology was successfully applied to detect xenobiotic-induced changes of this Ca^{2+} signal (Chapter VIII). Usually, the Ca^{2+} wave at fertilization is measured after microinjection of fluorescent Ca^{2+} indicator dyes (Poenie et al. 1985; Swann and Whitaker 1985) which is not only an elaborate technique requiring specialized

instruments but which may also damage the plasma membrane. Microinjection results in high dye loadings of cells which enable the measurement of small changes in intracellular Ca^{2+} concentrations. Nonetheless, the Ca^{2+} waves recorded in the present study are comparable to the ones measured in other studies with sea urchins using microinjected dyes (Chapter VIII).

However, at present we can only speculate on the underlying mechanisms which enabled us to record the fertilization Ca^{2+} wave with Fura-2 AM and MK571. Due to the large size of eggs, intracellular changes in Ca^{2+} are probably preliminary detectable in the cellular regions close to the plasma membrane. We hypothesize that Fura-2 is, in the absence of MK571, removed from the cytoplasmic spaces just underneath the egg plasma membrane and exported out of the cell. This might explain the absence of signals reported with Fura-2 in the absence of MK571. Confocal laser scanning microscopy should be applied to localize the dye Fura-2 in the absence as well as in the presence of MK571 during fertilization and elucidate the mechanism which enabled the measurement of the Ca^{2+} signal at fertilization.

Confocal laser scanning microscopy may further be useful to detect small changes in intracellular Ca^{2+} concentrations within eggs. Using epifluorescence microscopy there was no indication for xenobiotic-mediated changes of Ca^{2+} levels in unfertilized eggs in contrast to the alterations during fertilization in the present study. However, the Ca^{2+} signal at fertilization is a relatively strong cellular signal. Smaller changes in intracellular concentrations of Ca^{2+} may not be detected due to the large size of the eggs.

Furthermore, the present study provides perspectives for future studies regarding the toxicity of copper and TBP. Both substances were shown to enhance the Ca^{2+}-signal at fertilization. This may be the result of, for example, an increased permeability of the plasma membrane to Ca^{2+} as well as the release of sequestered Ca^{2+} from internal stores as previously shown by several authors (Pesando et al. 1991, Pesando et al. 1996, Girard, et al. 1997, Walter et al. 1989) for other chemicals in sea urchin eggs. Furthermore, investigations on Ca^{2+} content and uptake of Ca^{2+} as well as the role of mitochondrial damage may further shed more light on the toxicity of Cu^{2+} as these processes have previously shown to be involved in the toxicity of mercury chloride on sea urchin eggs (Walter et al. 1989).

Bromophenols have recently been shown to affect larval survival and metamorphosis of sea urchins (Agatsuma et al. 2008). Interestingly, TBP also inhibits fertilization and alters Ca^{2+}-signalling at fertilization in *P. miliaris* (chapter VII). As bromophenols are widely found in the

marine environment and at especially high concentrations in macroalgae (Howe et al. 2005) they have previously been proposed as a chemical defence and deterrence against grazers (Woodin et al. 1997; Kicklighter et al. 2004; Agatsuma et al. 2008). Another Ca^{2+}-mediated mechanism being essential for fertilization is the motility of sperm. The effects of Cu^{2+} on Ca^{2+} levels in sperm have previously been investigated by Earnshaw et al. (1986) in sperm of the mussel *Mytilus edulis.* Copper treatment significantly decreased Ca^{2+} levels in mitochondria and acrosomes possibly resulting in an increase in cytosolic Ca^{2+} in the sperm (Earnshaw et al. 1986). Nonetheless, to our knowledge there are no studies yet investigating sperm motility as well as Ca^{2+} signalling in sperm after exposure to bromophenols. Future research is needed for understanding the effects of brominated phenols on reproduction and recruitment of marine invertebrates in natural environments.

Although, there is still lack of knowledge concerning reproductive disorders of marine invertebrates caused by naturally occurring as well as anthropogenic chemicals, the experiments conducted using the echinoid *Psammechinus miliaris* form a promising basis for future studies not only on sea urchins but also on other invertebrates. Gonadal histopathology may, for example, be applied in biomonitoring provided that environmental toxicologists are sensitised to the seasonality as well as sex-specific susceptibility of these tissues. Ca^{2+}-signalling in sea urchins opens up new perspectives on fundamental cell biological research and both, gonadal histopathology as well as Ca^{2+}-signalling – are interesting for studies on the effects of endocrine disruptive compounds.

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Acknowledgements

It is without saying, that this work would not have been possible without the help of many people. I thank Prof. Dr. Angela Köhler for giving me the oppurtinity to conducting this thesis and for giving me the freedom to develop my research interest. I also thank her for supervising me throughout my thesis. I thank Prof. Dr. Klaudia Brix for her interest, time and effort as reviewer of my thesis. I thank Dr. Ulf Bickmeyer for completing my thesis comittee. I also thank Ulf for his collaboration on our publication on calcium signalling. I enjoyed having him as my coworker and working at the Biological Station on Helgoland. Thanks further to the members of my PhD committee at the Alfred Wegener Institute, Dr. Doris Abele and Prof. Allan Cembella, for their help, support and discussions. I also thank Doris and her working group, especially Ellen Weihe and Stefanie Meyer, for their collaboration.

I thank the working group *Cell Biology and Toxicology* for all the support during the last years. I especially would like to thank Sieglinde as the "heart" of our working group. Thanks to Sieglinde and Katja also for providing me a place to sleep in Bremerhaven during the stressful final times of my thesis. I thank the staff at the Biological Anstalt Helgoland for their help and friendliness during my research stays. I would further like to thank Anja Röw and Margret Krüß for maintaining the sea urchins at the BAH. I thank the crew of the MS Mya and Birgit Hussel at the Wadden Sea Station on Sylt for collecting the sea urchins. Without their help this thesis would not have been possible.

My trainees Yvonne Sakka and Thomas Langer I would like to thank for processing some of my samples so properly and diligently. It was fun having you around and passing on some of my knowledge. Thanks further to Lennart, Adilah, Alicja, Yetunde and Afua for their work during traineeships or lab rotations. I thank Steffi Gäbler-Schwarz for sharing our responsibilities as PhD student representatives.

I thank Prof. Dr. Thomas Brey for his help with the statistics and BoxCox-transformation of my data. I thank the staff of the AWI library for not getting tired organizing me all those papers via inter-library loan. I thank Christiane Schäfer from the Zeiss company for writing me all these Macros for the image analysis and for being such a good teacher in Axiovision. I thank Nicole, Manu, Sonja, Enken, Katja, Steffi and Ellen for critically reading my manuscripts and helping me to improve this thesis.

Furthermore, I am thankful for the support of my friends during these often stressful and hard times. It was great to have you around! Thank you for discussions, distractions and shared times! Last but not least, I deeply thank my parents for all their support, friendship and love.

Vielen Dank!

Declaration by Word of Honor

I, herewith, certify on my honor that this thesis is my own work and that I have completed it without undue help from third parties and without the use of any material other than permitted. Any thoughts and ideas taken directly or indirectly from others are highlighted as such. Neither this work in its present form nor any other work of its contents has been submitted to another German or foreign University.

Sabine Schäfer Bremen, 31st of July 2009