



**HAL**  
open science

## Gonadal lesions of female sea urchin ( after exposure to the polycyclic aromatic hydrocarbon phenanthrene

S. Schäfer, A. Köhler

► **To cite this version:**

S. Schäfer, A. Köhler. Gonadal lesions of female sea urchin ( after exposure to the polycyclic aromatic hydrocarbon phenanthrene. *Marine Environmental Research*, 2009, 68 (3), pp.128. 10.1016/j.marenvres.2009.05.001 . hal-00499562

**HAL Id: hal-00499562**

**<https://hal.science/hal-00499562>**

Submitted on 10 Jul 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

## Accepted Manuscript

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

S. Schäfer, A. Köhler

PII: S0141-1136(09)00053-1  
DOI: [10.1016/j.marenvres.2009.05.001](https://doi.org/10.1016/j.marenvres.2009.05.001)  
Reference: MERE 3338

To appear in: *Marine Environmental Research*

Received Date: 12 November 2008  
Revised Date: 29 April 2009  
Accepted Date: 7 May 2009



Please cite this article as: Schäfer, S., Köhler, A., Gonadal lesions of female sea urchin (*Psammechinus miliaris*) after exposure to the polycyclic aromatic hydrocarbon phenanthrene, *Marine Environmental Research* (2009), doi: [10.1016/j.marenvres.2009.05.001](https://doi.org/10.1016/j.marenvres.2009.05.001)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

3  
4  
5  
6  
7  
8 4 Schäfer S., Köhler A.\*

9  
10  
11  
12 6 *Alfred Wegener Institute for Polar and Marine Research in the Helmholtz Association, Am*  
13 *Handelshafen 12, 27570 Bremerhaven, Germany*

14  
15  
16  
17  
18  
19 9 \* Corresponding author

20  
21  
22  
23 11 Prof. Dr. Angela Köhler

24  
25 12 Phone: 0049 (0) 471 4831 1407

26  
27 13 Fax: 0049 (0) 471 4831 1425

28  
29 14 Email: [Angela.Koehler@awi.de](mailto:Angela.Koehler@awi.de)

30  
31  
32  
33  
34 16 Alfred Wegener Institute for Polar and Marine Research

35  
36 17 Am Handelshafen 12

37  
38 18 27570 Bremerhaven

39  
40 19 Germany

41  
42  
43  
44  
45  
46 21 **1. Abstract**

47  
48  
49 22 Histopathological alterations in the reproductive tissue of the female sea urchin  
50  
51 23 *Psammechinus miliaris* after exposure to the polycyclic aromatic hydrocarbon (PAH)  
52  
53 24 phenanthrene were investigated using light microscopy. Sexually mature sea urchins were  
54  
55 25 exposed to 500 µg L<sup>-1</sup> phenanthrene for 20 days. A severe disorganisation of the acinal  
56  
57 26 structure of the gonads was observed in phenanthrene-treated animals, mainly as a result of  
58  
59 27 aggregates of degenerating previtellogenic oocytes. Growth and maturation of previtellogenic

28 oocytes were inhibited, whereas there were no obvious effects on vitellogenic oocytes and  
29 mature ova. The acinal wall showed signs of fibrosis as well as dilation of the two epithelial  
30 layers. Phenanthrene severely affected the reproductive function of the sea urchins since a  
31 proper oogenesis of the ovarian tissue is very unlikely. Generally, the observed alterations  
32 are similar to histopathological changes in reproductive tissue in other invertebrates after  
33 exposure to PAHs and oil.

### 35 **Key words**

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

### 39 **2. Introduction**

40 Many environmental pollutants have been shown to interfere with reproductive function in  
41 animals (Haschek and Rousseaux, 1998). Sea urchin gametes and embryos are widely used  
42 for evaluating biological effects of contaminants in marine environments (Greenwood, 1980;  
43 Kobayashi, 1980; Hose, 1985; Klöckner et al., 1985; U.S. EPA, 1993). However, the  
44 underlying mechanisms resulting in reduced reproductive success of adult marine  
45 invertebrates and of sea urchins, in particular, are hardly investigated (Au et al., 2001a; Au et  
46 al., 2001b). Histopathology is a well established tool for the identification of tissue and  
47 cellular injury. Histopathological biomarkers and cellular changes in the reproductive tissue  
48 can be used to gain a better understanding of the adverse effects of environmental pollutants  
49 on reproduction (Au, 2004; Dietrich et al., in press).

50  
51 Sea urchins play a key role in benthic communities of coastal waters and changes in sea  
52 urchin populations have led to major alterations in marine community structure (Scheibling,  
53 1986; Steneck et al., 2002). *Psammechinus miliaris* is a common echinoid found at depths of  
54 up to 100 metres in the Atlantic from Scandinavia to the Azores including the North Sea and  
55 the Baltic Sea (Boschma et al., 1932; Campbell, 1977). This species has been used as a test

56 organism in fertilisation and embryo development assays (Klößner et al., 1985; Caldwell et  
1  
2 57 al., 2002; Caldwell et al., 2004). In the past years *P. miliaris* has gained interest as promising  
3  
4 58 candidate for aquaculture especially in polyculture with the Atlantic salmon (Kelly et al.,  
5  
6 59 1998).  
7

8  
9 60  
10  
11 61 Sea urchins are dioecious and due to their pentaradial body organisation they possess five  
12  
13 62 gonads with separate gonadopores (Westheide and Rieger, 1996). Functional units of the  
14  
15 63 gonads are the so-called acini which are surrounded by a multi-layered acinal wall (Palmer  
16  
17 64 Wilson, 1940). Germ cells are formed at the germinal epithelium of the acinal wall and  
18  
19 65 migrate towards the lumen of the acinus during maturation (e.g. Byrne, 1990; Walker et al.,  
20  
21 66 1998; Kelly, 2000). During oogenesis oocytes undergo meiotic divisions and an increase in  
22  
23 67 size to form mature ova. In the ovary oogenic cells, namely oogonia, previtellogenic and  
24  
25 68 vitellogenic oocytes can be distinguished from mature ova by the presence of the germinal  
26  
27 69 vesicle (GV) containing a prominent nucleolus (Chatlynne, 1969). Further, nutrients are  
28  
29 70 supposed to be transferred from the somatic cells of the gonads, the nutritive phagocytes  
30  
31 71 (NPs), to the gametogenic cells (Walker et al., 1998). Therefore, vitellogenic oocytes and  
32  
33 72 mature ova have - in contrast to the early maturational stages, the oogonia and  
34  
35 73 previtellogenic oocytes - yolk granules in their cytoplasm. In most sea urchin species living at  
36  
37 74 higher latitudes gametogenesis is a single annual event with one spawning period (Walker et  
38  
39 75 al., 1998). At the west coast of Scotland *Psammechinus miliaris* is reported to have a clearly  
40  
41 76 defined reproductive cycle with a single spawning period in June and July (Kelly, 2000).  
42  
43  
44  
45  
46  
47  
48

49 78 There are only few studies investigating the effects of chemicals on gonadal tissue of sea  
50  
51 79 urchins. At the light microscopical level histopathological lesions of sea urchin gonads are  
52  
53 80 reported by Vashchenko and Zhadan (1993), Vaschenko et al. (2001) and Vashchenko et al.  
54  
55 81 (2001) in areas of Peter the Great Bay (Sea of Japan) polluted with heavy metals.  
56  
57 82 Histopathological alterations of spermatogenesis in the sea urchin *Anthocidaris crassipina*  
58  
59  
60  
61  
62  
63  
64  
65

83 after experimental exposure to phenol and cadmium were investigated by electron  
84 microscopy (Au et al., 2001b; Au et al., 2003).

85

86 Polycyclic aromatic hydrocarbons (PAH) are lipophilic organic compounds which are  
87 ubiquitously found in the marine environment. Phenanthrene is a low molecular weight, 3-  
88 ring PAH and is classified as a priority pollutant by the United States Environmental  
89 Protection Agency (US EPA). It is an important petroleum-source PAH and a major  
90 component of the total content of PAH compounds in the marine environment. PAHs are  
91 known to induce degeneration of oocytes and ovarian lesions in several species (*Mytilus*  
92 *edulis* - Aarab et al., 2004; *Littorina littorea* - Cajaraville et al., 1990; mammals - Sakamaki,  
93 2003). Degeneration and absorption of oocytes, so called oocyte atresia, is a normal  
94 physiologic event which may become pathologic after chemical exposure (Blazer, 2002;  
95 Dietrich et al., in press). Furthermore, contamination with PAHs has been shown to be  
96 associated with an increased accumulation of lipofuscin in marine organisms (Krishnakumar  
97 et al., 1994; Krishnakumar et al., 1997; Au et al., 1999; Au, 2004). Lipofuscin, also known as  
98 age pigment, is widely regarded as end product of protein and lipid peroxidation due to  
99 oxidative stress (Au et al., 1999; Au, 2004; Terman and Brunk, 2004). In the sea urchin  
100 *Lythechinus variegatus* phenanthrene significantly retarded embryo development at  
101 environmentally relevant concentrations (Steevens et al., 1999). In *Lythechinus anemensis*  
102 phenanthrene disrupted axial development in embryos in a dose-dependent fashion which  
103 could be linked to nuclear accumulation of the protein  $\beta$ -catenin (Pillai et al., 2003). Pillai et  
104 al. (2003) have shown that among five investigated PAHs phenanthrene was most potent in  
105 causing exogastrulation in sea urchin embryos.

106

107 The aim of the present study was to give a detailed description of the histopathological  
108 alterations of the reproductive tissue of the female sea urchin *Psammechinus miliaris* after  
109 exposure to phenanthrene. The findings are compared with effects of hydrocarbons on the  
110 reproductive tissue of other aquatic invertebrates. Changes of the general acinal structure,

111 effects on the different developmental stages of oocytes and the nutritive phagocytes, as well  
112 as alterations of the acinal wall are described. Lipofuscin is identified and quantified in the  
113 tissue and its usefulness as biomarker of pollutant exposure in the gonads of sea urchins is  
114 discussed. Histopathological alterations of the gonads such as aggregates of degenerating  
115 oocytes, the percentage of degenerating oocytes, lipofuscin accumulation and dilation of the  
116 acinal wall are quantitatively or semi quantitatively assessed. Furthermore, the gonad weight  
117 and the gonadosomatic index in the different treatments are compared.

### 118 **3. Material & Methods**

#### 119 **3.1 Animal collection and experimental set up**

120 Adult *Psammechinus miliaris* were collected with a beam trawl close to the Island of Sylt  
121 (Germany) at 55° 02, 40 N and 08° 27, 25 E in May 2006. They were separated by sex and  
122 transported to the laboratory in Bremerhaven. In a range finding experiment, sea urchins  
123 were exposed to set concentrations of 1, 3, 12, 42, 145 and 500 µg L<sup>-1</sup> phenanthrene, to  
124 0.01% acetone, as the solvent control, and to sea water only for 10 days. Each treatment  
125 consisted of two replicate tanks (glass vessels with a volume of 1.5 L) with 4 sea urchins  
126 each. Since no mortality occurred in this preliminary experiment 500 µg L<sup>-1</sup> phenanthrene  
127 was chosen as sublethal test concentration for the subsequent experiment. Female sea  
128 urchins of a minimum size of 20 mm horizontal test diameter were selected and acclimatized  
129 to experimental conditions for 4 weeks. Sea urchins were exposed to 500 µg L<sup>-1</sup>  
130 phenanthrene (dissolved in acetone), to 0.01% acetone, as solvent control, and to sea water  
131 only (9 ± 0.5 °C, 33 ± 1 PSU, continuous aeration). Duration of the experiment was limited to  
132 20 days to terminate the test before the natural spawning period of the animals had started.  
133 Each treatment consisted of 3 replicate tanks (16 L glass aquaria) containing 10 sea urchins  
134 each. Water was renewed every third day and phenanthrene or solvent were added at the  
135 respective concentrations. Sea urchins were fed with fresh spinach. Mortality of sea urchins  
136 was checked daily.

### 139 3.2 Sampling

1  
2 140 For comparison of growth parameters, 24 samples were taken at the beginning (day 0) and  
3  
4 141 30 replicate samples per treatment were taken at the end of the exposure period (day 20).  
5  
6 142 Animals were weighed and horizontal and vertical test diameters were measured with a  
7  
8 143 calliper. The animals were dissected and the gonads were removed and weighed. The gonad  
9  
10  
11 144 index (GI) was calculated as the wet weight of the gonad divided by the total somatic wet  
12  
13 145 weight of the sea urchin expressed as a percentage.  
14

15 146

16  
17 147 For histopathology, six replicate samples were taken per treatment at day 20. Gonads were  
18  
19  
20 148 immediately fixed in Baker's Formol calcium (4% formaldehyde and 2% calcium acetate)  
21  
22 149 over night. On the next day, the samples were transferred into gum sucrose (30% sucrose  
23  
24 150 and 1% gum arabicum) and stored at 4°C.  
25

26 151

### 28 152 3.3 Embedding, sectioning and staining

29  
30  
31 153 Gonad samples were dehydrated with an increasing series of acetone of 70% and 100% and  
32  
33 154 embedded in methacrylate as described elsewhere (Köhler, 2004). After the embedding  
34  
35 155 procedure, tissue blocks were left to dry for at least two days and serial sections of 2 µm  
36  
37 156 thickness were cut on a microtome (HM Leica RM 2145).  
38  
39

40 157

41  
42 158 The sections were stained with hematoxylin and eosin (H&E) for demonstration of  
43  
44 159 eosinophilic and basophilic structures, with Alcian Blue/PAS for neutral and acidic  
45  
46 160 mucopolysaccharides (glycosaminoglycans) such as hyaluronic acid, chondroitin sulfate and  
47  
48  
49 161 sialic acids, with Schmorl's staining procedure for lipofuscin, and with Picrosirius for collagen.  
50

51 162

52  
53 163 Most H&E staining procedures proved to be insufficient possibly due to the low eosinophilia  
54  
55 164 of the tissue. The most appropriate method was the one described by Cerri and Cerri-Sassi  
56  
57  
58 165 (2003) which is appropriate for methacrylate embedded tissue.  
59

60 166



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

178

179 For the Schmorl's procedure sections were stained according to Pearse (1985) with minor  
180 modifications: Sections were brought to water, stained in staining solution (freshly prepared  
181 0.5% ferric chloride and 0.5 % potassium ferricyanide in distilled water) for 15 minutes,  
182 washed in 1% acetic acid for 2 min., washed in running tap water for 10 min. and rinsed  
183 again in distilled water. Slides were left to dry and mounted in Euparal. A staining time of 15  
184 minutes proved to be optimal and resulted in blue staining of lipofuscin granules. Longer  
185 staining resulted in background staining and staining of lipochromes present in yolk platelets.

186

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

190

### 191 3.4 Microscopy

192 Slides were viewed with a microscope (Axioscope, ZEISS) and images were taken with a  
193 camera (MRc, ZEISS) coupled to a computer equipped with the software AxioVision (Vers.  
194 4.6.3.0, ZEISS).

195 Tissue sections stained with the Schmorl's method were quantitatively and objectively  
196 assessed for lipofuscin content using computer assisted image analysis. The image analysis  
197 consisted of the above described microscope and camera and the software KS300 (Vers.  
198 3.0, ZEISS). Image intensity was measured using grey values from 1 (clear, white) to 255  
199 (dark, black). Three black and white images were randomly taken from each duplicate  
200 section of gonadal tissue (6 measurements per individual) with a 10x objective. In a special  
201 macro individual ovarian acini were marked as 'region of interest'. Lipofuscin granules within  
202 the defined acini were identified with the aid of a threshold function which discriminates the  
203 darker stained lipofuscin granules from the background. The software computed the areal  
204 density of lipofuscin [%] within the marked acinus ('region of interest') and mean values for  
205 each individual were calculated.

206 Further quantifications were performed on sections stained with Alcian Blue/PAS method.  
207 Aggregates of degenerating oocytes were quantified by applying an eyepiece graticule with a  
208 100 squares grid (10 x 10) as an overlay. The number of squares lying on the acinus and the  
209 number of squares lying on aggregates of degenerating oocytes were counted at 400x  
210 magnification. In each duplicate section five areas were randomly chosen (10 measurements  
211 per animal). The areal density of oocyte aggregates [%] within the acini was calculated.  
212 Dilation of the acinal wall was semi quantitatively assessed. For this purpose, in each sample  
213 5 randomly chosen areas were graded according to a scaling scheme from 0 (no change) to  
214 2 (severe alteration) at 100 x magnification. Degenerating previtellogenic oocytes identified  
215 by fading and vacuolisation of the cytoplasm, cell lysis and disruption of the membrane of the  
216 germinal vesicle at 600 x magnification were counted. Oogonia were not considered due to  
217 their small size and the resultant difficulty in diagnosis. 100 randomly selected previtellogenic  
218 oocytes were counted per sample and the data are presented as a percentage.

219

### 220 3.5 Statistics

221 Residuals were tested for normality and variance homogeneity (Levene's test). Data on  
222 gonad weight and gonad index were box cox-transformed and a one-way ANOVA with Tukey

223 HSD as post-hoc test were run. The data for aggregates of degenerating oocytes and  
1 lipofuscin content were square-root transformed. For the data on histopathology a one-way  
2 224 ANOVA was run with the Holm-Sidak method as post-hoc test. In case of non-normal data a  
3  
4 225 Kruskal-Wallis ANOVA on ranks with a Dunn's test as post-hoc test was used. The  
5  
6 226 significance level was set at  $p < 0.05$ . For the growth data tests were run with JMP (Version  
7  
8 227 7.0, SAS Institute Inc.) while histopathological analysis was done with Sigma Stat (Version  
9  
10 228 3.0.1, SPSS, 1992-2003).  
11  
12 229  
13  
14 230

## 17 231 **4. Results**

### 19 232 **4.1 Gonad weight and gonad index**

20 233 During the 20 day exposure period no mortality occurred among the test animals. Individual  
21  
22 234 gonad weight was highly variable in all treatments (Table 1). Yet, gonad weight as well as  
23  
24 235 gonad index were significantly reduced after phenanthrene exposure. In the solvent control  
25  
26 236 gonad weight was also reduced but not significantly different from controls. Somatic size  
27  
28 237 (horizontal and vertical test diameters) and somatic weight were not different at the beginning  
29  
30 238 and at the end of the experiment as well as between treatments.  
31  
32  
33  
34  
35 239

### 36 240 **4.2 Alterations of the general gonad structure**

37 241 In control animals, acini of the gonads were characterized by the presence of oocytes and  
38  
39 242 nutritive phagocytes (NP) surrounded by a relatively thin multilayered acinal wall (Figure 1 a).  
40  
41 243 Oocytes at different developmental stages were present with the oogonia and previtellogenic  
42  
43 244 oocytes (Figure 1 b) preferably found close to the acinal wall and the vitellogenic oocytes  
44  
45 245 (Figure 1 c) and mature ova (Figure 1 d) in the lumen of the acini. The occurrence and  
46  
47 246 frequency of the different oogenic stages varied depending on the reproductive stage of the  
48  
49 247 animal as well as in the different acini of each individual. The NPs formed a meshwork inside  
50  
51 248 of the acini which was more or less visible depending on the reproductive stage of the animal  
52  
53 249 and the treatment.  
54  
55  
56  
57  
58  
59  
60 250  
61  
62  
63  
64  
65

251 At the beginning of the experiment (day 0) test animals (n=10) were in the premature stage  
1  
2 252 according to the classification of the reproductive stages by Byrne (1990) and Kelly (2000).  
3  
4 253 The animals were partly spawned which was observed by empty spaces which had been  
5  
6 254 occupied by oocytes before. At the end of the experiment (day 20) the sea urchins in the  
7  
8 255 three treatments were still, except of one control animal, in the premature stage and partially  
9  
10 256 spawned (Figure 1 a). The one control animal was in the recovery period.  
11  
12

13 257  
14  
15 258 In phenanthrene-exposed animals, the acinal structure was severely affected: Early  
16  
17 259 developmental stages of oocytes were no longer found close to the acinal wall but spread in  
18  
19 260 the acinal lumen, the meshwork of NPs appeared disrupted and aggregates of degenerating  
20  
21 261 previtellogenic oocytes were found in the acini (Figure 2 a, b). Control animals hardly showed  
22  
23 262 any aggregates of degenerating oocytes while in phenanthrene-exposed animals these  
24  
25 263 aggregates filled large areas of most of the acini. The areal density of these aggregates was  
26  
27 264 significantly increased in phenanthrene-treated animals in comparison to the control and the  
28  
29 265 solvent control groups (one-way ANOVA,  $p = 0.006$ , Figure 3 a). Remarkably, less  
30  
31 266 vitellogenic oocytes were found in the gonads of animals exposed to phenanthrene in  
32  
33 267 comparison to controls.  
34  
35  
36  
37

38 268  
39  
40 269 Lipofuscin identified as irregularly shaped and heterogeneously textured blue granules in  
41  
42 270 sections stained with the Schmorl's technique was found in the acini (Figure 4 a, b). High  
43  
44 271 amounts of lipofuscin were localized in extracellular spaces in the acini and inside of NPs  
45  
46 272 (Figure 4 a, b). In addition, lipofuscin was found in epithelial cells of the acinal wall and in  
47  
48 273 conjunction with immune cells, the so called coelomocytes, outside of the acini (Figure 4 a).  
49  
50 274 No lipofuscin was observed in oocytes. Indeed, early developmental stages of oocytes can  
51  
52 275 hardly be identified in sections stained with the Schmorl's technique due to their small size  
53  
54 276 and the lack of a counterstain. Lipofuscin deposition in oogonia and previtellogenic oocytes  
55  
56 277 can, therefore, not be excluded. Overall, lipofuscin content was increased but not  
57  
58  
59  
60  
61  
62  
63  
64  
65

278 significantly different in phenanthrene-treated animals (one-way ANOVA,  $p = 0.068$ , Figure 3  
1  
2 279 b).

3  
4 280

5  
6 281 4.3 Effects on germ and somatic cells

7  
8 282 In all treatments, oogonia, previtellogenic and vitellogenic oocytes as well as mature ova  
9  
10 283 were found in the gonads. Previtellogenic oocytes are usually localized close to the acinal  
11  
12 284 wall. Morphologically, they are characterized by a large germinal vesicle, a high nucleus to  
13  
14 285 cytoplasmic ratio and their small size in comparison to later developmental stages. Their  
15  
16 286 cytoplasm is stained blue in H&E and Alcian Blue/PAS (Figure 1 b). Vitellogenic oocytes, on  
17  
18 287 the other hand, accumulate PAS+ material in the cytoplasm with ongoing maturation visible  
19  
20 288 as pink granules in the Alcian Blue/PAS stained sections. Vitellogenic oocytes are larger and  
21  
22 289 their nucleus to cytoplasmic ratio is reduced in comparison to previtellogenic oocytes (Figure  
23  
24 290 1 c). They also possess a large germinal vesicle which breaks down to form ripe ova (Figure  
25  
26 291 1 d) accumulating in the acinal lumen of mature animals.  
27  
28  
29  
30

31 292  
32  
33 293 We could not detect pathological changes of mature ova and vitellogenic oocytes while  
34  
35 294 previtellogenic oocytes were often degenerating after maternal exposure to phenanthrene.  
36  
37 295 Degenerating oocytes were characterized by fading and vacuolisation of the cytoplasm as  
38  
39 296 well as cell lysis. Further, infoldings of the nuclear membrane of the GV occurred which was  
40  
41 297 followed by disruption of the nuclear membrane (Figure 2 c). The nucleolus appeared to be  
42  
43 298 normal. No pyknosis or karyomegaly of the GV was identified. In comparison to control  
44  
45 299 samples, cell size of previtellogenic oocytes seemed to be reduced. Oogonia were not  
46  
47 300 studied in detail due to their small size and the resultant difficulty in diagnosis of cellular  
48  
49 301 alterations.  
50

51 302  
52  
53 303 Degenerating previtellogenic oocytes identified by fading and vacuolisation of the cytoplasm,  
54  
55 304 cell lysis and / or infoldings and disruption of the nuclear membrane were quantified in the  
56  
57 305 gonadal tissue (Figure 3 c). In phenanthrene-treated animals 78 % of the previtellogenic  
58  
59  
60  
61  
62  
63  
64  
65

306 oocytes were classified as degenerating based on the above mentioned criteria, whereas in  
1  
2 307 controls 24 % degenerating previtellogenic oocytes were found. In the solvent control group  
3  
4 308 the frequency of degenerating oocytes was quite variable with a few animals exhibiting high  
5  
6 309 amounts of degenerating oocytes. However, differences were only significant between the  
7  
8 310 control group and phenanthrene-treated animals (one-way ANOVA,  $p = 0.01$ , Holm-Sidak).

10 311  
11  
12 312 As mentioned above, the meshwork of NPs was disrupted after exposure to phenanthrene.  
13  
14 313 Moreover, in phenanthrene-exposed animals the NPs appeared to be more detached from  
15  
16 314 each other and engaged in phagocytotic activity. No karyomegaly, nuclear polymorphism, or  
17  
18 315 nuclear inclusions were observed in NPs. Yolk was identified as globules of various shades  
19  
20 316 of pink and blue in nutritive phagocytes in the Alcian Blue/PAS stain.  
21  
22  
23

#### 24 317 25 26 318 4.4 Alterations of the acinal wall

27  
28 319 The acinal wall is usually composed of an inner and an outer layer of epithelial cells which  
29  
30 320 are characterized by their basophilic nuclei. Depending on the plane of the tissue section at  
31  
32 321 least one additional layer is visible being stained pink with H&E as well as with the Picrosirius  
33  
34 322 method indicating the presence of collagen fibres (Figure 4 c).  
35  
36

37 323  
38  
39 324 In phenanthrene-exposed animals the two epithelial layers were often separate from each  
40  
41 325 other (Figure 4 d, e). Often this dilation was concomitant with an increased formation of  
42  
43 326 collagen between the epithelial layers and augmentation of basophilic material (Figure 4 d).  
44  
45 327 Histological scoring shows that phenanthrene exposure resulted in a significantly increased  
46  
47 328 dilation of the acinal wall (one-way ANOVA,  $p < 0.001$ , Holm-Sidak, Figure 3 d). In solvent-  
48  
49 329 treated samples dilation of the acinal wall occurred slightly more often than in control  
50  
51 330 samples.  
52  
53

54 331

#### 55 332 5. Discussion

333 It is well known that PAHs and oil elicit histopathological effects on gonads in marine  
1  
2 334 invertebrates such as degeneration of germ cells and gonadal follicles, atrophy of gonadal  
3  
4 335 tissue as well as inflammatory reactions (Lowe and Pipe, 1986; Berthou et al., 1987;  
5  
6 336 Cajaraville et al., 1990; Tay et al., 2003; Aarab et al., 2004; Ortiz-Zarragoitia and Cajaraville,  
7  
8 337 2006). Indeed, the mentioned studies deal solely with molluscan species. To our knowledge  
9  
10  
11 338 the present study is the first one investigating the effects of a PAH on sea urchin gonads.  
12

13 339  
14  
15 340 *Psammechinus miliaris* showed severe ovarian lesions after exposure to the sublethal  
16  
17 341 concentration of phenanthrene. The gonad size and the gonad index of the sea urchins were  
18  
19 342 significantly reduced indicating tissue atrophy. Similarly, Berthou et al. (1987) observed  
20  
21 343 atrophy of the gonad concomitantly with a decrease in the gonad index in oysters in areas  
22  
23 344 polluted by the Amoco Cadiz oil spill.  
24  
25

26 345  
27  
28 346 Strikingly, in *P. miliaris* exposed to phenanthrene the acinal structure was disorganized which  
29  
30 347 was mainly due to aggregates of degenerating oocytes. This disorganisation makes a proper  
31  
32 348 oogenesis unlikely since the migration of maturing oocytes towards the acinal lumen is  
33  
34 349 hampered. Generally, degeneration of ovarian follicles is described as follicular atresia and  
35  
36 350 stress such as exposure to xenobiotics appears to increase the incidence of atretic follicles  
37  
38 351 (Dietrich et al., in press). Degenerating follicles were also observed in *Mytilus edulis* exposed  
39  
40 352 to a mixture of North Sea oil, alkylphenols and PAHs. Exposure to 0.5 ppm North Sea oil  
41  
42 353 alone, however, increased the size and number of ovarian follicles in the mussels (Aarab et  
43  
44 354 al., 2004). In the present study, a few degenerating acini were also found in control animals  
45  
46 355 which may resorb malfunctional oocytes to preserve normal gonadal function as known from  
47  
48 356 atretic follicles in female fish (Dietrich et al., in press).  
49  
50

51 357  
52  
53 358 Furthermore, phenanthrene exposure significantly increased degeneration of previtellogenic  
54  
55 359 oocytes in the ovary of *P. miliaris*. PAHs and oil are known to increase the prevalence of  
56  
57 360 oocyte degeneration or oocyte atresia in molluscs: Ortiz-Zarragoitia and Cajaraville (2006)  
58  
59  
60  
61  
62  
63  
64  
65

361 found a higher volume density of atretic oocytes in mussels (*Mytilus edulis*) exposed to 0.5  
1 362 ppm North Sea oil and to a mixture of North Sea oil (0.5 ppm), alkylphenols (0.1 ppm) and  
2 363 extra polycyclic aromatic hydrocarbons (0.1 ppm) for three weeks. In *M. edulis*  
3 364 experimentally exposed to low (28 ppb) and high (128 ppb) doses of diesel oil for three  
4 365 months the volume of atretic oocytes was also significantly increased (Lowe and Pipe, 1986).  
5  
6 366 In the Baltic clam (*Macoma balthica*) necrosis of primary and secondary oocytes as well as  
7 367 ripe ova was reported after 28 days exposure to sediments highly contaminated with PAHs,  
8 368 polychlorinated biphenyls (PCBs), and heavy metals (Tay et al., 2003). Astonishingly, in  
9 369 clams exposed to sediment with a very high concentration of PAHs no ovarian lesions were  
10 370 detected (Tay et al., 2003).  
11  
12 371

13 372 In the present study, previtellogenic oocytes showed a reduction in size and vitellogenic  
14 373 oocytes showed a reduction in numbers in *P. miliaris* after maternal exposure to  
15 374 phenanthrene. It is unclear whether inhibition of oocyte growth and maturation by  
16 375 phenanthrene exposure is the underlying mechanism of these findings. The low number of  
17 376 vitellogenic oocytes may also be a result of spawning induced by exposure to phenanthrene  
18 377 since spawning is known to be accelerated in mussels (*Mytilus galloprovincialis*) exposed to  
19 378 high doses of oil (Cajaraville et al., 1992). However, no increased spawning events in the  
20 379 phenanthrene-treated group were observed during the experiment in comparison to controls.  
21 380 Regarding the previtellogenic oocytes, it could be speculated that their small size may, next  
22 381 to an inhibition of growth, result from degeneration and their resorption by NPs. Studying the  
23 382 effects of phenanthrene exposure at different times during oogenesis may clarify whether  
24 383 phenanthrene exposure inhibits growth and maturation of oocytes. Interestingly, in contrast  
25 384 to the severe alterations of previtellogenic oocytes, we found no adverse effects of  
26 385 phenanthrene on vitellogenic oocytes and mature eggs. It has to be considered that later  
27 386 developmental stages of oocytes may be better protected against pollutant stress: Eggs of  
28 387 sea urchins and sea stars are known to express low levels of efflux transporter genes  
29 388 (Hamdoun et al., 2004; Roepke et al., 2006). In sea stars efflux transporter activities are  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65



389 known to increase during oocyte maturation (Roepke et al., 2006). Moreover, younger  
1  
2 390 oocytes may be more sensitive to pollutants due to their higher surface to volume ratio.  
3  
4 391 Further, it has to be considered that release of affected gametes may result in  
5  
6 392 underestimating the adverse effects of phenanthrene on oocytes and eggs in *P. miliaris*.  
7

8  
9 393  
10  
11 394 After exposure to phenanthrene we observed elevated but not statistically significant  
12  
13 395 increased deposition of lipofuscin in the ovaries of *P. miliaris*. In several studies, lipofuscin  
14  
15 396 accumulation in fish liver and mussel digestive gland is associated with oxidative stress  
16  
17 397 caused by pollution with oil and PAHs (Krishnakumar et al., 1994, 1997; Au et al. 1999; Au,  
18  
19 398 2004). In the reproductive tissue of the sea urchin *Strongylocentrotus intermedius* inhabiting  
20  
21 399 polluted areas in the Sea of Japan, lipofuscin accumulation was related to contamination with  
22  
23 400 heavy metals (Vashchenko and Zhadan, 1993; Vaschenko et al., 2001; Vashchenko et al.,  
24  
25 401 2001). As demonstrated in the present study, Vaschenko et al. (2001) and Vashchenko et al.  
26  
27 402 (2001) also found lipofuscin deposition in NPs and in the hemal sinuses of the acinal walls.  
28  
29 403 However, in contrast to our findings, the authors further identified lipofuscin accumulation in  
30  
31 404 germ cells of *S. intermedius* (Vaschenko et al., 2001; Vashchenko et al., 2001). Indeed,  
32  
33 405 preliminary studies with a wild population of *P. miliaris* indicate that lipofuscin accumulation in  
34  
35 406 the reproductive tissue is dependent on the reproductive stage and the sex of the organism  
36  
37 407 (Schäfer & Köhler, unpublished). It has to be further investigated whether lipopigment  
38  
39 408 deposition in reproductive tissue of sea urchins is a useful biomarker for pollutant stress.  
40  
41  
42  
43  
44  
45

46 409  
47 410 The NPs showed increased phagocytic activity in the phenanthrene-treated animals. This  
48  
49 411 may indicate early phagocytosis and resorption of gonadal and oocyte material as a result of  
50  
51 412 improper ovarian and gamete development. Gamete resorption is a natural phenomenon in  
52  
53 413 echinoderms that occurs in both sexes at the end of the spawning season (Kalachev and  
54  
55 414 Reunov, 2005) but it is also described in the sea urchin *Strongylocentrotus intermedius* from  
56  
57 415 polluted areas in the Sea of Japan (Vashchenko and Zhadan, 1993). The PAH-induced  
58  
59  
60  
61  
62  
63  
64  
65

416 gamete resorption in the gonads is also reported in the prosobranch *Littorina littorea* after  
1 short-term exposure to high concentrations of the PAH 1-naphthol (Cajaraville et al., 1990).  
2  
3

4 418  
5  
6 419 The acinal wall of *Psammechinus miliaris* was severely affected by phenanthrene exposure  
7 showing dilation of the epithelial layers and increased formation of collagen. Further,  
8 420 augmentation of basophilic structures occurred which can not be identified so far. The  
9 thickness of the gonadal wall of the sea urchin *Arbacia punctulata* is reported to change  
10 during the year (Palmer Wilson, 1940). Increased deposition of collagen in conjunction with a  
11 421 thickened appearance of connective tissue, however, is generally known as fibrosis (Dietrich  
12 et al., in press). In reproductive tissue of invertebrates fibrosis is described from gonadal  
13 422 neoplasms in mussels (Peters et al., 1994). And in the prosobranch *Littorina littorea* fibrosis  
14 was shown after short-term exposure to high concentrations of the PAH 1-naphthol  
15 423 (Cajaraville et al., 1990). When immune cells are present simultaneously fibrosis is assumed  
16 to be a scarring process. In a few studies infiltration with immune cells have been described  
17 424 in invertebrates after exposure to oil and PAHs (Berthou et al., 1987; Cajaraville et al., 1990;  
18 Cajaraville et al., 1992). Indeed, a clear diagnosis of immune reactions in the present study  
19 425 was difficult since, to our knowledge, there is no detailed histological description of immune  
20 cells of sea urchins, the coelomocytes, *in situ*. Furthermore, coelomocytes had a similar  
21 426 appearance as epithelial cells of the acinal wall in *Psammechinus miliaris*.  
22  
23  
24 427  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

436 The concentration of phenanthrene used in the present study is, in general, higher than  
437 levels of PAHs found in the marine environment: In seawater around England and Wales  
438 background concentrations of PAHs range from none detected to 10.7  $\mu\text{g L}^{-1}$  total PAHs  
439 (Law et al., 1997). 'Safe' levels of phenanthrene for aquatic organisms set by several  
440 countries are equal or below 4.6  $\mu\text{g L}^{-1}$  (Law et al., 1997). After the Exxon Valdez oil spill in  
441 Prince William Sound in the Northern Gulf of Alaska in 1989 where around 37 000 metric  
442 tons of crude oil were released an average of 0.4  $\mu\text{g L}^{-1}$  total petroleum hydrocarbon were  
443 measured at polluted sites (Boehm et al., 1990). However, after the Hebei Spirit incident at

444 the West coast of Korea in July 2007 up to 17 mg L<sup>-1</sup> and an average of 626 µg L<sup>-1</sup> total  
1 petroleum hydrocarbons were measured (Shim, 2008). However, direct comparison of  
2 445 concentrations used in the present study and levels of PAHs or phenanthrene found in the  
3 446 marine environment in general or at point sources are difficult since combinations of PAHs  
4 447 may have different effects than single chemicals (Hylland, 2006). Contamination of sea  
5 448 urchin habitats with PAHs e.g. by oil spills or release of oil from oil platforms may have  
6 449 severe impacts on sea urchin populations. Daan & Mulder (1996), for example, have shown  
7 450 that the abundance of the echinoidea *Echinocardium cordatum* was reduced close to a  
8 451 drilling station in the North Sea where oil-based drilling muds had been used. Following the  
9 452 Torrey Canyon oil spill in 1967 large numbers of dead *Psammechinus miliaris* were found in  
10 453 the vicinity which may be attributed to the hydrocarbon exposure and the heavy spraying of  
11 454 hydrocarbon based dispersants in that area (Smith, 1968).  
12 455

13 456  
14 457 In summary, 20 days exposure towards sublethal concentrations of phenanthrene resulted in  
15 458 severe ovarian lesions of *Psammechinus miliaris* which make a proper oogenesis unlikely.  
16 459 Strikingly, the acinal structure was disorganized which may be analogous to follicular atresia  
17 460 in other invertebrates and fish. Previtellogenic oocytes were severely affected while there  
18 461 were no obvious effects on vitellogenic oocytes and mature ova. For future studies, the  
19 462 usefulness of lipofuscin accumulation in gonads of sea urchins as a biomarker needs to be  
20 463 further investigated. Moreover, a detailed description of coelomocytes *in situ* is necessary to  
21 464 clearly identify immune reactions in sea urchin tissues and to compare histopathological  
22 465 effects of pollutants with studies on other invertebrate species.  
23 466

## 51 467 **6. Acknowledgements**

52 468 This study was financed by the Alfred Wegener Institute for Polar and Marine Research  
53 469 within the Helmholtz Association. It is part of a PhD study conducted at the Jacobs University  
54 470 Bremen. The authors thank B. Hüssel and the staff of the FS Mya for collecting the sea  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

471 urchins. We are grateful to two anonymous reviewers for providing helpful comments on the  
1  
2 472 manuscript.  
3

4 473

5  
6 474 **7. Literature**

7  
8  
9 475 Aarab, N., Minier, C., Lemaire, S., Unruh, E., Hansen, P.D., Larsen, B.K., Andersen, O.K.,  
10 476 Narbonne, J.F., 2004. Biochemical and histological responses in mussel (*Mytilus edulis*)  
11 477 exposed to North Sea oil and to a mixture of North Sea oil and alkylphenols. Marine  
12 478 Environmental Research 58, 437-441.  
13 479

14 480 Au, D.W.T., 2004. The application of histo-cytopathological biomarkers in marine pollution  
15 481 monitoring: a review. Marine Pollution Bulletin 48, 817-834.  
16 482

17 483 Au, D.W.T., Lee, C.Y., Chan, K.L., Wu, R.S.S., 2001a. Reproductive impairment of sea  
18 484 urchins upon chronic exposure to cadmium. Part I: Effects on gamete quality. Environmental  
19 485 Pollution 111, 1-9.  
20 486

21 487 Au, D.W.T., Reunov, A.A., Wu, R.S.S., 2001b. Reproductive impairment of sea urchin upon  
22 488 chronic exposure to cadmium. Part II: Effects on sperm development. Environmental  
23 489 Pollution 111, 11-20.  
24 490

25 491 Au, D.W.T., Wu, R.S.S., Zhou, B.S., Lam, P.K.S., 1999. Relationship between ultrastructural  
26 492 changes and EROD activities in liver of fish exposed to Benzo[a]pyrene. Environmental  
27 493 Pollution 104, 235-247.  
28 494

29 495 Au, D.W.T., Yurchenko, O.V., Reunov, A.A., 2003. Sublethal effects of phenol on  
30 496 spermatogenesis in sea urchins (*Anthocidaris crassispina*). Environmental Research 93, 92-  
31 497 98.  
32 498

33 499 Berthou, F., Balouet, G., Bodennec, G., Marchand, M., 1987. The occurrence of  
34 500 hydrocarbons and histopathological abnormalities in oysters for 7 Years Following the Wreck  
35 501 of the Amoco-Cadiz in Brittany (France). Marine Environmental Research 23, 103-133.  
36 502

37 503 Blazer, V.S., 2002. Histopathological assessment of gonadal tissue in wild fishes. Fish  
38 504 Physiology and Biochemistry 26, 85-101.  
39 505

40 506 Boehm, P.D., Neff, J.M., Page, D.S., 2007. Assessment of polycyclic aromatic hydrocarbon  
41 507 exposure in the waters of Prince William Sound after the Exxon Valdez oil spill: 1989-2005.  
42 508 Marine Pollution Bulletin 54, 339-367.  
43 509

44 510 Boschma, H., de Beaufort, L.F., van Oort, E.D., Redeke, H.C., Roepke, W., 1932. Aflevering  
45 511 VI: Echinodermata. A. W. Sijthoff's Uitgeversmij N.V., Leiden.  
46 512

47 513 Byrne, M., 1990. Annual reproductive cycles of the commercial sea urchin *Paracentrotus*  
48 514 *lividus* from an exposed intertidal and a sheltered subtidal habitat on the west coast of  
49 515 Ireland. Marine Biology 104, 275-289.  
50 516

51 517 Cajaraville, M.P., Marigómez, J.A., Angulo, E., 1990. Short-term toxic effects of 1-naphthol  
52 518 on the digestive gland-gonad complex of the marine prosobranch *Littorina littorea* (L): A light  
53 519 microscopic study. Archives of Environmental Contamination and Toxicology 19, 17-24.  
54 520

55 521 Cajaraville, M.P., Marigómez, J.A., Angulo, E., 1992a. Comparative effects of the water  
56 522 accommodated fraction of three oils on mussels - 1. Survival, growth and gonad  
57  
58  
59  
60  
61  
62

- 523 development. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology  
1 524 102, 103-112.
- 2 525
- 3 526 Cajaraville, M.P., Marigomez, J.A., Diez, G., Angulo, E., 1992b. Comparative Effects of the  
4 527 Water Accommodated Fraction of Three Oils on Mussels - 2. Quantitative Alterations in the  
5 528 Structure of the Digestive Tubules. Comparative Biochemistry and Physiology Part C:  
6 529 Comparative Pharmacology 102, 113-123.
- 7 530
- 8 531 Caldwell, G.S., Bentley, M.G., Olive, P.J.W., 2004. First evidence of sperm motility inhibition  
9 532 by the diatom aldehyde 2E, 4E-decadienal. Marine Ecology Progress Series 273, 97-108.
- 10 533
- 11 534 Caldwell, G.S., Olive, P.J.W., Bentley, M.G., 2002. Inhibition of embryonic development and  
12 535 fertilization in broadcast spawning marine invertebrates by water soluble diatom extracts and  
13 536 the diatom toxin 2-trans,4-trans decadienal. Aquatic Toxicology 60, 123-137.
- 14 537
- 15 538 Campbell, A.C., 1977. The Hamlyn Guide to the seashore and shallow seas of Britain &  
16 539 Europe. Hamlyn Publishing Group, London, 320 pp.
- 17 540
- 18 541 Cerri, P.S., Sasso-Cerri, E., 2003. Staining methods applied to glycol methacrylate  
19 542 embedded tissue sections. Micron 34, 365-372.
- 20 543
- 21 544 Chatlynne, L.G., 1969. A histochemical study of oogenesis in the sea urchin  
22 545 *Strongylocentrotus purpuratus*. Biological Bulletin 136, 167-184.
- 23 546
- 24 547 Daan, R. & Mulder, M. 1996. On the short-term and long-term impact of drilling activities in  
25 548 the Dutch sector of the North Sea. ICES Journal of Marine Science 53: 1036-1044.
- 26 549
- 27 550 Dietrich, D.R., Krieger, H.O., Rumpf, S., in press. Histological Analysis of endocrine  
28 551 disruptive effects in small laboratory fish. Wiley & Sons Inc.
- 29 552
- 30 553 Greenwood, P.J., 1980. Possible uses of the developmental embryology of the sea urchin  
31 554 *Parechinus angulosus* for pollution monitoring in the nearshore marine environment. PhD  
32 555 thesis, University of Cape Town, 334 pp.
- 33 556
- 34 557 Hamdoun, A.M., Cherr, G.N., Roepke, T.A., Epel, D., 2004. Activation of multidrug efflux  
35 558 transporter activity at fertilization in sea urchin embryos (*Strongylocentrotus purpuratus*).  
36 559 Developmental Biology 276, 452-462.
- 37 560
- 38 561 Haschek, W.M., Rousseaux, C.G., 1998. Fundamentals of Toxicologic Pathology. Academic  
39 562 Press, San Diego. 563 pp.
- 40 563
- 41 564 Hose, J.E., 1985. Potential uses of sea urchin embryos for identifying toxic chemicals:  
42 565 description of a bioassay incorporating cytologic, cytogenetic and embryologic endpoints.  
43 566 Journal of Applied Toxicology 5, 245-254.
- 44 567
- 45 568 Hylland, K. 2006. Polycyclic aromatic hydrocarbon (PAH) ecotoxicology in marine  
46 569 ecosystems. Journal of Toxicology and Environmental Health A 59, 109-123.
- 47 570
- 48 571 Kalachev, A.V., Reunov, A.A., 2005. Resorption of Gametes in the Testes of the Sea Star  
49 572 *Asterina pectinifera* (Mueller et Troschel, 1842). Russian Journal of Marine Biology 31, 119-  
50 573 123.
- 51 574
- 52 575 Kelly, M.S., 2000. The reproductive cycle of the sea urchin *Psammechinus miliaris*  
53 576 (Echinodermata: Echinoidea) in a Scottish sea loch. Journal of the Marine Biological  
54 577 Association of the UK 80, 909-919.
- 55 578

- 579 Kelly, M.S., Brodie, C.C., McKenzie, J.D., 1998. Somatic and gonadal growth of the sea  
1 580 urchin *Psammechinus miliaris* (Gmelin) maintained in poyculture with the Atlantic salmon.  
2 581 Journal of Shellfish Research 17, 1557-1562.  
3 582
- 4 583 Klöckner, K., Rosenthal, H., Willführ, J., 1985. Invertebrate bioassays with North Sea water  
5 584 samples. I. Structural effects on embryos and larvae of serpulids, oysters and sea urchins.  
6 585 Helgoland Marine Research 39, 1-19.  
7 586
- 8 587 Kobayashi, N., 1980. Comparative sensitivity of various developmental stages of sea urchins  
9 588 to some chemicals. Marine Biology 58, 163-171.  
10 589
- 11 590 Köhler, A., 2004. The gender-specific risk to liver toxicity and cancer of flounder (*Platichthys*  
12 591 *flesus* (L.)) at the German Wadden Sea coast. Aquatic Toxicology 70, 257-276.  
13 592
- 14 593 Krishnakumar, P.K., Casillas, E., Varanasi, U., 1994. Effect of environmental contaminants  
15 594 on the health of *Mytilus edulis* from Puget Sound, Washington, USA. 1. Cytochemical  
16 595 measures of lysosomal responses in the digestive cells using automatic image analysis.  
17 596 Marine Ecology Progress Series 106, 249-261.  
18 597
- 19 598 Krishnakumar, P.K., Casillas, E., Varanasi, U., 1997. Cytochemical responses in the  
20 599 digestive tissue of *Mytilus edulis* complex exposed to microencapsulated PAHs or PCBs.  
21 600 Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and  
22 601 Endocrinology 118, 11-18.  
23 602
- 24 603 Law, R.J., Dawes, V.J., Woodhead, R.J., Matthiessen, P. 1997. Polycyclic aromatic  
25 604 hydrocarbons (PAH) in seawater around England and Wales. Marine Pollution Bulletin 34(5),  
26 605 306-322.  
27 606
- 28 607 Lendrum, A.C., Slidders, W., Fraser, D.S., 1972. Renal hyalin - Study of amyloidosis and  
29 608 diabetic fibrinous vasculosis with new staining methods. Journal of Clinical Pathology 25,  
30 609 373.  
31 610
- 32 611 Lowe, D.M., Pipe, R.K., 1986. Hydrocarbon exposure in mussels - a quantitative study of the  
33 612 responses in the reproductive and nutrient storage cell systems. Aquatic Toxicology 8, 265-  
34 613 272.  
35 614
- 36 615 Ortiz-Zarragoitia, M., Cajaraville, M., 2006. Biomarkers of exposure and reproduction-related  
37 616 effects in mussels exposed to endocrine disruptors. Archives of Environmental  
38 617 Contamination and Toxicology 50, 361-369.  
39 618
- 40 619 Palmer Wilson, L., 1940. Histology of the gonad wall of *Arbacia punctulata*. Journal of  
41 620 Morphology 66, 463-479.  
42 621
- 43 622 Pearse, A.G.E. 1985. Histochemistry: Theoretical and Applied, Vol. 2, Analytical Technology,  
44 623 4th edition, Churchill Livingstone, Edinburgh, London, Melbourne and New York, p. 748.  
45 624
- 46 625 Peters, E.C., Yevich, P.P., Harshbarger, J.C., Zaroogian, G.E., 1994. Comparative  
47 626 histopathology of gonadal neoplasms in marine bivalve mollusks. Diseases of Aquatic  
48 627 Organisms 20, 59-76.  
49 628
- 50 629 Pillai, M.C., Vines, C.A., Wikramanayake, A.H., Cherr, G.N., 2003. Polycyclic aromatic  
51 630 hydrocarbons disrupt axial development in sea urchin embryos through a [beta]-catenin  
52 631 dependent pathway. Toxicology 186, 93-108.  
53 632  
54 633  
55 634  
56 635

- 633 Roepke, T.A., Hamdoun, A.M., Cherr, G.N., 2006. Increase in multidrug transport activity is  
1 634 associated with oocyte maturation in sea stars. *Development Growth and Differentiation* 48,  
2 635 559-573.
- 3 636
- 4 637 Sakamaki, K., 2003. Physiological and pathological cell deaths in the reproductive organs.  
5 638 *Cell Structure and Function* 28, 31-40.
- 6 639
- 7 640 Scheibling, R., 1986. Increased macroalgal abundance following mass mortalities of sea  
8 641 urchins (*Strongylocentrotus droebachiensis*) along the Atlantic coast of Nova-Scotia.  
9 642 *Oecologia* 68, 186-198.
- 10 643
- 11 644 Schäfer, S. & Köhler, A., unpublished. Seasonal alterations in reproductive tissue of the sea  
12 645 urchin *Psammechinus miliaris* in the northern Wadden Sea.
- 13 646
- 14 647 Shim, W.K. 2008. Environmental impact assessment following the *Hebei Spirit* incident.  
15 648 KORDI-NOAA workshop. Korean Ocean Research and Development Institute (KORDI).  
16 649 Ansan. Korea. 13<sup>th</sup> of May 2008
- 17 650
- 18 651 Smith, J.E., 1968. 'Torrey Canyon'. Pollution and marine life. A report by the Plymouth  
19 652 Laboratory of the Marine Biological Association of the United Kingdom. Cambridge.  
20 653 Cambridge University Press.
- 21 654
- 22 655 Steevens, J.A., Slattery, M., Schlenk, D., Aryl, A., Benson, W.H., 1999. Effects of ultraviolet-  
23 656 B light and polyaromatic hydrocarbon exposure on sea urchin development and bacterial  
24 657 bioluminescence. *Marine Environmental Research* 48, 439-457.
- 25 658
- 26 659 Steneck, R.S., Graham, M.H., Bourque, B.J., Corbett, D., Erlandson, J.M., Estes, J.A.,  
27 660 Tegner, M.J., 2002. Kelp forest ecosystems: biodiversity, stability, resilience and future.  
28 661 *Environmental Conservation* 29, 436-459.
- 29 662
- 30 663 Tay, K.L., Teh, S.J., Doe, K., Lee, K., Jackman, P., 2003. Histopathologic and histochemical  
31 664 biomarker responses of Baltic clam, *Macoma balthica*, to contaminated Sydney Harbour  
32 665 sediment, Nova Scotia, Canada. *Environmental Health Perspectives* 111, 273-280.
- 33 666
- 34 667 Terman, A., Brunk, U.T., 2004. Lipofuscin. *International Journal of Biochemistry & Cell*  
35 668 *Biology* 36, 1400-1404.
- 36 669
- 37 670 U.S. EPA, 1993. Sea urchin, *Arbacia punctulata*, fertilization test, Method 1008.0. In: Short-  
38 671 term methods for estimating the chronic toxicity of effluents and receiving waters to marine  
39 672 and estuarine organisms, 2nd ed. US Environmental Protection Agency, report EPA/600/4-  
40 673 91/003, July 1994, Cincinnati, OH, pp 300-340
- 41 674
- 42 675 Vaschenko, M.A., Zhadan, P.M., Latypova, E.V., 2001. Long-term changes in the state of  
43 676 gonads in sea urchins *Strongylocentrotus intermedius* from Amur Bay, the Sea of Japan.  
44 677 *Russian Journal of Ecology* 32, 358-364.
- 45 678
- 46 679 Vashchenko, M.A., Zhadan, P.M., 1993. Ecological assessment of marine environment using  
47 680 two sea urchin tests: disturbance of reproduction and sediment embryotoxicity. *Science of*  
48 681 *the Total Environment* 134, 1235-1245.
- 49 682
- 50 683 Vashchenko, M.A., Zhadan, P.M., Latypova, E.V., 2001. Long-term changes in the gonad  
51 684 condition of the sea urchin *Strongylocentrotus intermedius* from Alekseev Bight (Amurskii  
52 685 Bay) under polluted conditions. *Russian Journal of Marine Biology* 27, 49-53.
- 53 686
- 54 687
- 55 688
- 56 689
- 57 690
- 58 691
- 59 692
- 60
- 61
- 62
- 63
- 64
- 65

687 Walker, C.W., McGinn, N.A., Harrington, L.M., Lesser, M.P., 1998. New perspectives on sea  
1 688 urchin gametogenesis and their relevance to aquaculture. Journal of Shellfish Research 17,  
2 689 1507-1514.

3 690  
4 691 Westheide, W., Rieger, R., 1996. Spezielle Zoologie. Erster Teil: Einzeller & Wirbellose Tiere  
5 692 Gustav Fischer Verlag, Stuttgart, Jena, New York.

6 693  
7 694  
8

9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

ACCEPTED MANUSCRIPT



695 **8. Tables & Figures**

1 696

2 697 **8.1 Table**

3 698

4 699 Table 1

5 700

6 701 Growth parameters of *Psammecinus miliaris* in the different treatments. Standard deviations  
7 702 are in parentheses. Different letters in the same row indicate statistical significant differences  
8 703 between the treatments (control at day 0: n = 24, all other treatments: n = 30, one-way  
9 704 ANOVA, Tukey HSD,  $p < 0.05$ ).

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57

58

59

60

61

62

63

64

65

705

1 706 8.2 Figures

2 707

## 3 708 Figure 1

4 709

5 710 Ovary sections of control animals of *Psammechinus miliaris* stained with Alcian Blue/PAS a)  
 6 711 Typical acinus of an ovary surrounded by a thin acinal wall. Early oocyte stages such as  
 7 712 previtellogenic oocytes are found close to the acinal wall, whereas later oocyte stages such  
 8 713 as vitellogenic oocytes are found in the lumen of the acinus. Note the empty spaces between  
 9 714 the oocytes which result from spawning of oocytes. (bar = 100  $\mu\text{m}$ ) b) Previtellogenic oocyte  
 10 715 of a control animal with a large germinal vesicle containing a big nucleolus (bar = 10  $\mu\text{m}$ ), c)  
 11 716 vitellogenic oocyte showing PAS+ granules in the cytoplasm (bar = 20  $\mu\text{m}$ ), d) mature ova  
 12 717 showing PAS+ granules in the cytoplasm and missing a germinal vesicle (bar 20  $\mu\text{m}$ ). AW =  
 13 718 acinal wall, GV = germinal vesicle, N = nucleolus, NP = nutritive phagocytes, PO =  
 14 719 previtellogenic oocyte, VO = vitellogenic oocyte.

16 720

17 721

18 722

## 19 723 Figure 2

20 724

21 725 a) – c) Ovary sections of phenanthrene-exposed *Psammechinus miliaris* stained with Alcian  
 22 726 Blue/PAS a) acinus showing severe disorganisation of the acinal structure with an aggregate  
 23 727 of degenerating oocytes in the lower right and several small degenerating oocytes. Note the  
 24 728 dilation of the acinal wall. (bar = 100  $\mu\text{m}$ ) b) aggregate of degenerating oocytes (bar = 20  
 25 729  $\mu\text{m}$ ) c) oocytes showing signs of degeneration with vacuolisation of the cytoplasm, cell lysis,  
 26 730 infoldings of the nuclear membrane and disruption of the nuclear membrane (bar = 10  $\mu\text{m}$ ).  
 27 731 AL = acinal lumen, AW = acinal wall, D = disruption of the nuclear membrane, DO =  
 28 732 degenerating oocyte, GV = germinal vesicle, IF = infoldings of the nuclear membrane, Ly =  
 29 733 cell lysis, N = nucleolus, NP = nutritive phagocytes, PO = previtellogenic oocyte, VC =  
 30 734 vacuolisation of the cytoplasm.

31 735

32 736

## 33 737 Figure 3

34 738 Histopathologic alterations depicted as bar charts with the mean values and standard  
 35 739 deviations. Different letter indicate significant differences ( $p < 0.05$ ). a) areal density [%] of  
 36 740 aggregates of degenerating oocytes (one-way ANOVA, Holm-Sidak), b) areal density [%] of  
 37 741 lipofuscin accumulation in the ovaries (one-way ANOVA), c) percentage of degenerating  
 38 742 previtellogenic oocytes (Kruskal-Wallis, Dunn's test), d) dilation of the acinal wall (one-way  
 39 743 ANOVA, Holm-Sidak).

40 744

41 745

## 42 746 Figure 4

43 747 a) – b) Ovary section of a phenanthrene-exposed individual stained with the Schmorl's  
 44 748 method, a) overview taken at lower magnification showing high deposition of lipofuscin in the  
 45 749 acini. Note the lipofuscin accumulation outside of the acini in conjunction with coelomocytes  
 46 750 (bar = 100  $\mu\text{m}$ ), b) detail of a) with lipofuscin granules (arrows) (bar = 10  $\mu\text{m}$ ), c) – d) Ovary  
 47 751 sections of *Psammechinus miliaris* stained with Picrosirius, c) Thin acinal wall of a control  
 48 752 animal with the inner and outer epithelial layers (bar = 20  $\mu\text{m}$ ), d) Acinal wall of a  
 49 753 phenanthrene-exposed animal showing dilation of the inner and outer epithelial layers  
 50 754 concomitant with increased deposition of collagen and unknown basophilic material. Note the  
 51 755 small cells at the outer epithelial layer which may be attacking coelomocytes (bar = 20  $\mu\text{m}$ ),  
 52 756 e) Acinal wall of a phenanthrene-exposed animal showing dilation of the inner and outer  
 53 757 epithelial layers (bar = 20  $\mu\text{m}$ ). AW = acinal wall, bas+ = basophilic material, C = collagen,  
 54 758 CA = coelomocyte aggregate, CO = coelomocytes (?), DO = degenerating oocyte, GV =  
 55 759 germinal vesicle, IL = inner epithelial layer, N = nucleolus, NP = nutritive phagocytes, OL =  
 56 760 outer epithelial layer, PO = previtellogenic oocyte.

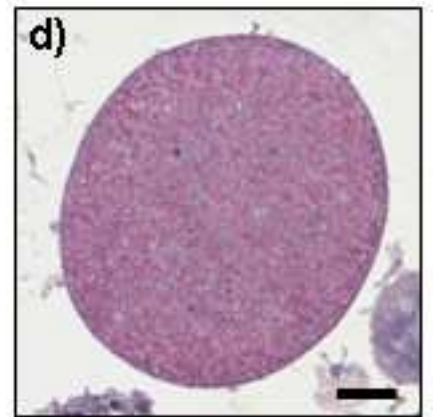
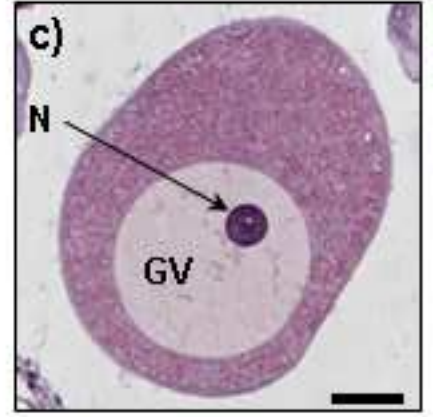
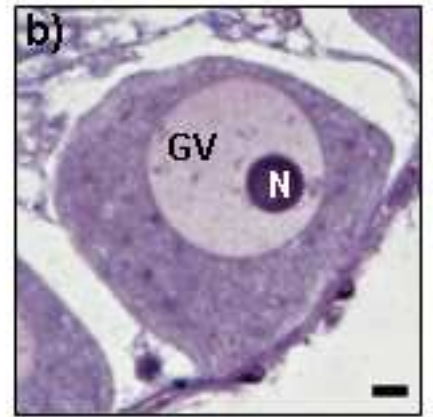
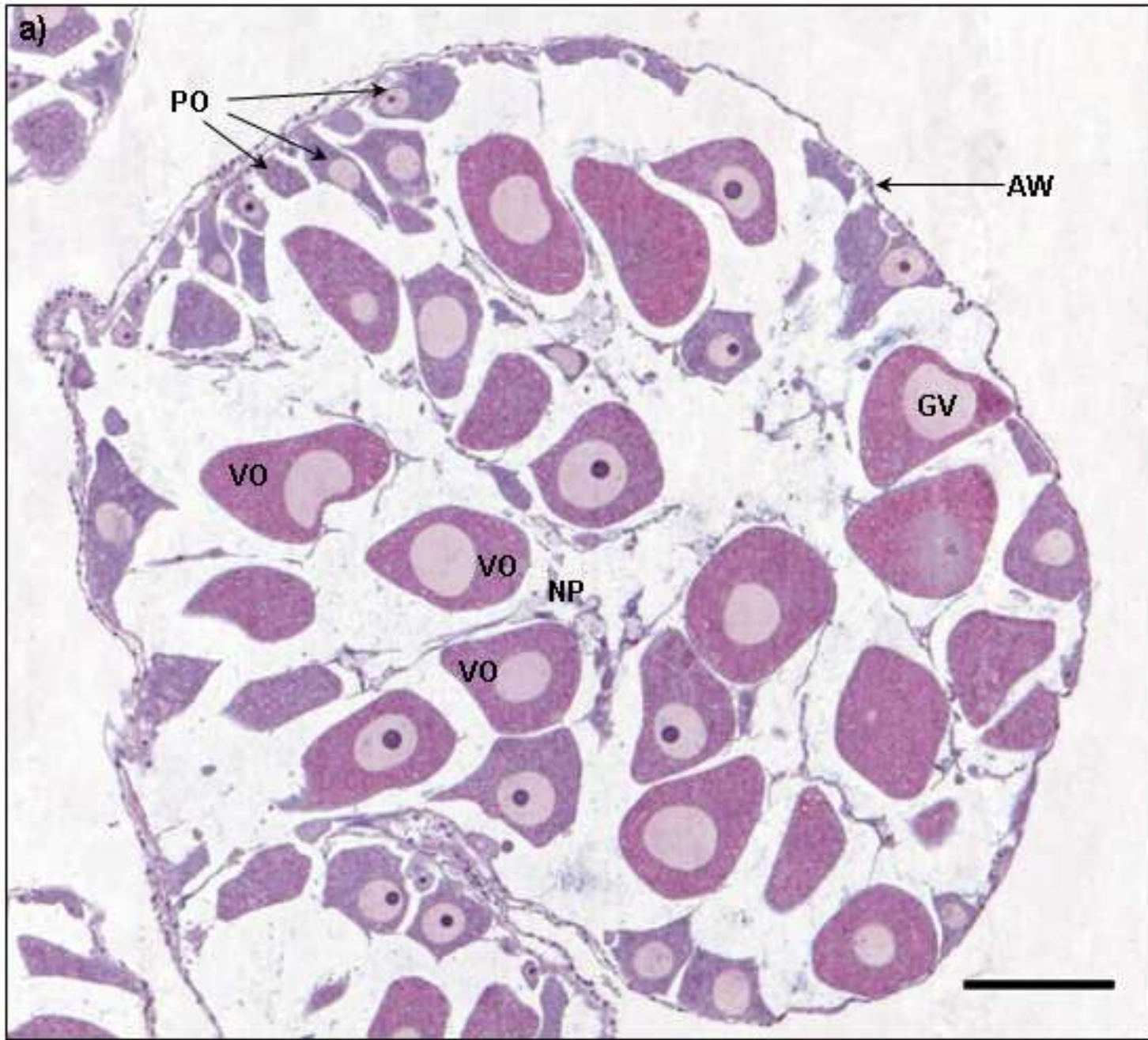
62

63

64

65

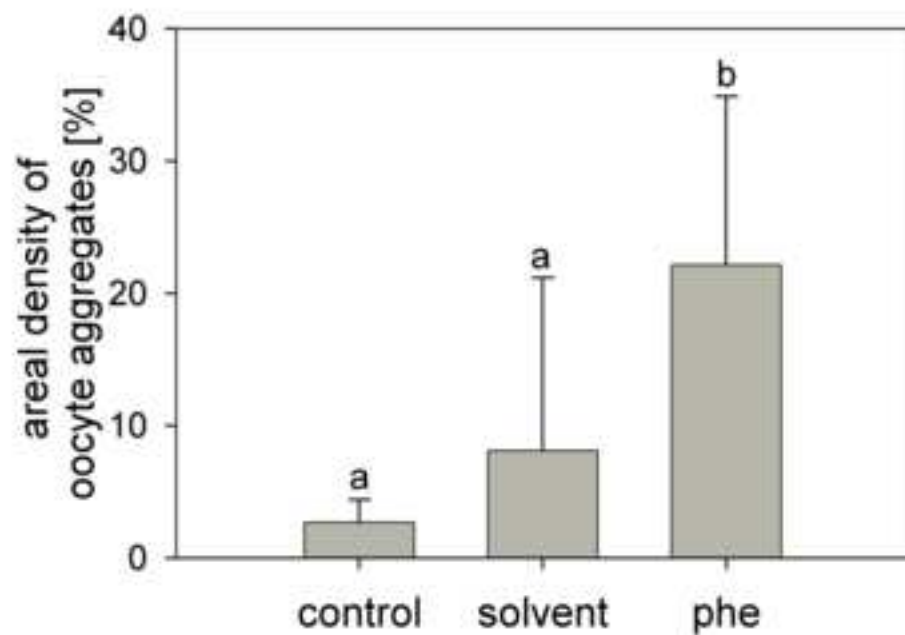
	days			
	0		20	
parameter / treatment	control	control	solvent control	phenanthrene
mean vertical test diameter [mm]	17.5 <sup>a</sup> (1.7)	17.4 <sup>a</sup> (1.9)	17.6 <sup>a</sup> (2.5)	17.4 <sup>a</sup> (2.8)
mean horizontal test diameter [mm]	30.9 <sup>a</sup> (3.2)	30.4 <sup>a</sup> (3.3)	30.6 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



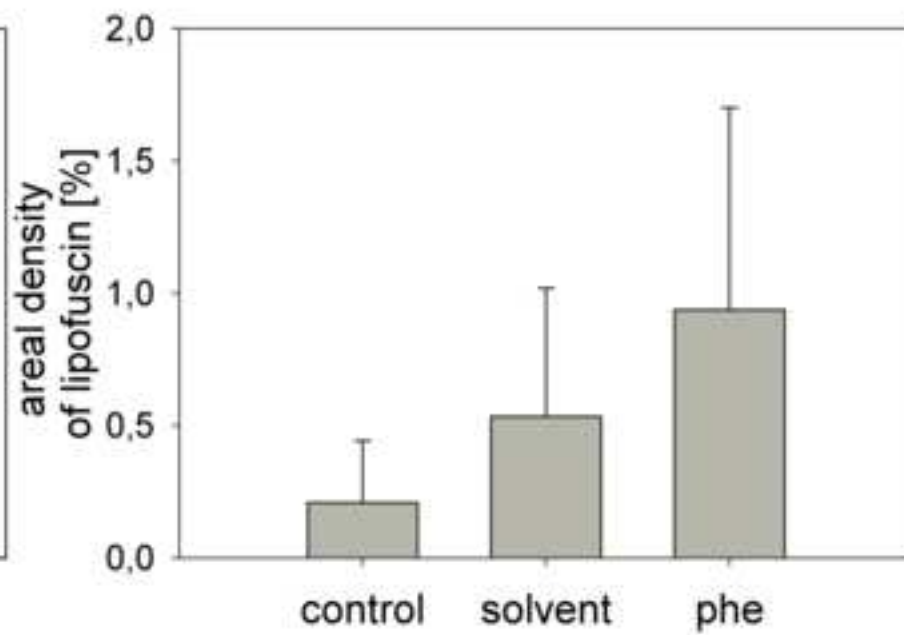




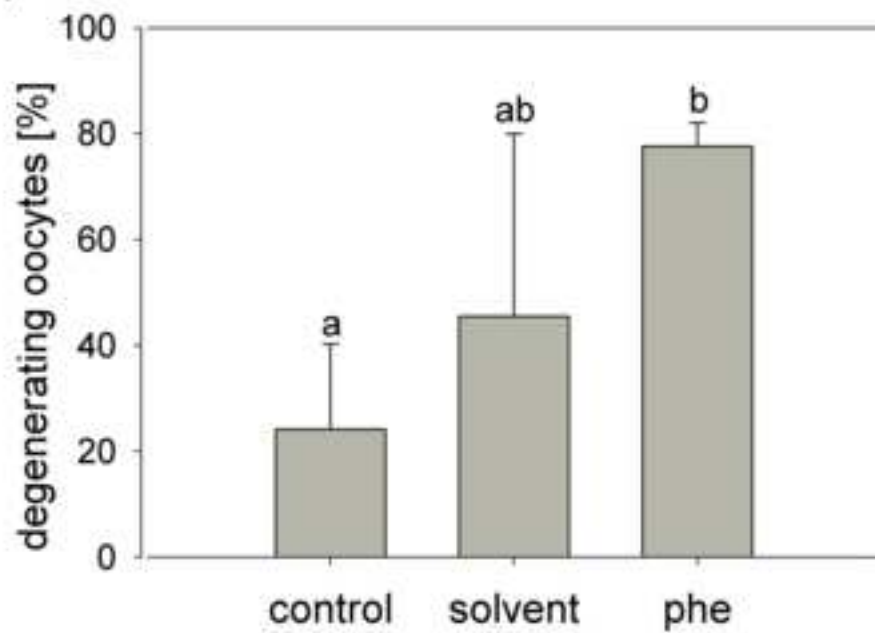
a)



b)



c)



d)

