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Effects of Endocrine Disruptors on Prosobranch Snails (Mollusca: Gastropoda) in the Laboratory. Part I: Bisphenol A and Octylphenol as Xeno-Estrogens

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Abstract. The effects of suspected endocrine disrupting chemicals on freshwater and marine prosobranch species were analysed in laboratory experiments. In this first publication, the responses of the freshwater snail *Marisa cornuarietis* and of the marine prosobranch *Nucella lapillus* to the xeno-estrogenic model compounds bisphenol A (BPA) and octylphenol (OP) are presented at nominal concentration ranges between 1 and 100 μg/L. *Marisa* was exposed during 5 months using adult specimens and in a complete life-cycle test for 12 months. In both experiments, the xeno-estrogens induced a complex syndrome of alterations in female *Marisa* referred to as "superfemales" at the lowest concentrations. Affected specimens were characterised by the formation of additional female organs, an enlargement of the accessory pallial sex glands, gross malformations of the pallial oviduct section resulting in an increased female mortality, and a massive stimulation of oocyte and spawning mass production. The effects of BPA and OP were comparable at the same nominal concentrations. An exposure to OP resulted in inverted U-type concentration response relationships for egg and spawning mass production. Adult *Nucella* from the field were tested for three months in the laboratory. As in *Marisa*, superfemales with enlarged accessory pallial sex glands and an enhancement of oocyte production were observed. No oviduct malformations were found probably due to species differences in the gross anatomical structure of the pallial oviduct. A lower percentage of exposed specimens had ripe sperm stored in their vesicula seminalis and additionally male *Nucella* exhibited a reduced length of penis and prostate gland when compared to the control. Because statistically significant effects were observed at the lowest nominal test concentrations (1 μg BPA or OP/L), it can be assumed that even lower concentrations may have a negative impact on the snails. The results show that prosobranchs are sensitive to endocrine disruption at environmentally relevant concentrations and that especially *M. cornuarietis* is a promising candidate for a future organismic invertebrate model to identify endocrine-mimetic test compounds.

Keywords: endocrine disruptors; xeno-estrogens; bisphenol A; octylphenol; snails

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Introduction

Recent reports have shown that a number of xenobiotics in the environment are capable of interfering with the normal endocrine function in a variety of animals and also in humans. Some of the reported effects of suspected endocrine disruptors in humans include decreased sperm counts, increased cases of breast, testicular and other forms of reproductive cancers, genital abnormalities (e.g. hypospadia, cryptorchidism), premature puberty in females, and increased cases of endometriosis (Gist, 1998). The overwhelming majority of the studies on the effects of hormone-mimetic industrial chemicals were focussed on findings in vertebrates. More detailed information about the effects on and mechanisms of action in invertebrates has only been obtained from a few cases although invertebrates represent more than 95% of the known species in the animal kingdom (deFur *et al*., 1999). The limited number of examples for endocrine disruption in invertebrates is partially due to the fact that their hormonal systems are rather poorly understood in comparison with vertebrates. Deleterious endocrine changes following an exposure to certain compounds may therefore easily be missed or simply be unmeasurable at present, even though a number of field investigations and laboratory studies show that endocrine disruption has probably occurred (for review: deFur *et al*., 1999). The example of tributyltin (TBT) compounds and their masculinising effects in about 150 species of prosobranch molluscs shows that apparently trivial biochemical changes (inhibition of aromatase activity according to Bettin *et al*. (1996), can have drastic effects up to the population and community levels by a final sterilisation of affected females. According to Matthiessen and Gibbs (1998) there is no reason to suppose that such far-reaching changes are in any sense unique. The main endocrine effects of TBT in these molluscs are the induction of imposex, an additional formation of male sex characters like penis and/or vas deferens on females (Gibbs *et al*., 1987; Oehlmann, 1994) and of intersex which is characterised by a modification or supplanting of female by male sex organs (Bauer *et al*., 1995, 1997).

This publication is the first in a series which investigates effects of compounds suspected to act as endocrine modulators on freshwater and marine prosobranch species in the laboratory. Most of the results were obtained during a research project for the German Federal Environmental Agency (project code 216 02 001/04) between September 1997 and May 2000. The objective was to develop an organismic invertebrate test system for the simultaneous identification of either androgen- or estrogen-mimicking chemicals. Recently, gonochoristic prosobranchs were rated as the most promising candidates for this purpose next to insects and crustaceans (deFur *et al*., 1999). This first publication is focussed on the effects of two suspected xeno-estrogens, bisphenol A (BPA) and octylphenol (OP). The next papers will be dedicated to the xeno-androgen triphenyltin and the xeno-antiandrogen vinclozolin.

The first evidence that bisphenol A and alkylphenols could be estrogenic was published in the 1930s on the basis of feeding experiments with BPA and 4-propylphenol to ovariectomised rats (Dodds and Lawson, 1936, 1938). More recent research has highlighted the implications of these effects. The growth of human breast cancer cell (MCF-7) cultures is affected by octylphenol at concentrations as low as 0.1 μM (20 μg/L) (e.g. Soto *et al*., 1991). Estrogenic effects have also been shown in tissue and cell culture experiments with rainbow trout hepatocytes, chicken embryo fibroblasts and in mammalian estrogen receptor assays (Jobling and Sumpter, 1993; White *et al*., 1994; Yamakoshi *et al*., 2000).

Bisphenol A is manufactured for the plastics industry as an intermediate in the production of polycarbonate and epoxy resins. A smaller amount is used in the manufacture of thermopaper, tyres, dental composites and sealants (BUA, 1997). The total BPA consumption in Germany was approximately 163,000 t in 1994. In 1993, the corresponding amounts for Western Europe were 347,000 t, for the USA 552,000 t and Japan 214,000 t (BUA, 1997).

BPA is not readily biodegradable (less than 1% transformation in 28 days in sewage treatment plants according to Howard, 1989) although with sufficiently adapted microorganisms, it can be eliminated to more than 90% in the laboratory and in industrial sewage treatment plants. Under

environmental conditions neither hydrolysis nor photolysis is likely (BUA, 1997).

There is evidence from estrogen receptor (Greim, 1998) and MCF-7 assays (Krishnan *et al*., 1993) that BPA exhibits an estrogen-mimetic action at concentrations as low as 2–5 μg/L. The results for in vivo studies with mammals are conflicting (see Discussion).

The occurrence of BPA in aquatic ecosystems is summarised by Rippen (1999). In effluents from sewage treatment plants in Berlin (Germany) concentrations of up to 160 ng/L were detected in 1997 (mean: 80 ng/L; median: 60 ng/L; n = 12). The highest concentrations in rivers were reported from Japan (Tokyo region) with values between 10 and 1,900 ng/L. In smaller rivers near Berlin concentrations of up to 410 ng/L were reported for 1997 (mean: 23 ng/L; median: 6 ng/L; n = 41). In the same river system sediments were contaminated with < 5–150 μg BPA/kg (dry wt.) (mean and median: 42 μg/kg; n = 19). In the Rhine estuary (The Netherlands) concentrations were between < 10 and 119 ng/L in 1989. The measured bioconcentration factors for BPA in carp (< 100), calculated values (BCF up to 366) and the log P_{ow} value of 3.32–3.4 suggest a low bioaccumulation potential in aquatic organisms (BUA, 1997).

Nonylphenol (NP) and octylphenol are the most important high production volume alkylphenols with nonylphenol ethoxylates (NPnEO) taking approximately 80% of the world market, and octylphenol ethoxylates (OPnEO) representing the remaining 20% (White *et al*., 1994). The alkylphenol ethoxylates are used in a variety of industrial processes, including wool washing, but no longer in domestic detergents in the EU. These compounds are biodegraded by removal of ethoxy groups, producing less biodegradable products, including alkylphenols like nonyl- and octylphenol which frequently persist through sewage treatment and in rivers (Ahel *et al*., 1994a,b). The estimated annual consumption of alkylphenol ethoxylates was 13,500 t in Germany in 1986 (BUA, 1991) and 18,500 t in the UK in 1992 (CES, 1993).

The total emission of octylphenol into U.K. waters was estimated to be about 300 kg in 1998 (Environment Agency, 1998). Rivers in Switzerland have been found to contain concentrations of tens of μg/L of a wide range of alkylphenolic compounds (Ahel *et al*., 1994b). Low levels were reported for drinking water in the USA, with a total concentration of alkylphenols of almost 1 μg/L and a concentration of OP2EO of 32 ng/L (Clark *et al*., 1992).

Materials and methods

The experiments were performed with two different gonochoristic prosobranch species, the freshwater ramshorn snail, *Marisa cornuarietis* (Mesogastropoda: Ampullariidae), and the marine dogwhelk *Nucella lapillus* (Neogastropoda: Muricidae). *Marisa* specimens came from our own laboratory breeding stock which was built up with specimens obtained from the breeding stock of Aquazoo Düsseldorf (Germany) in 1991. Dogwhelks came directly from the field and were collected at Méan Mélen, Brittany, in March 1999.

For all laboratory experiments, a 24 h (weekends: 48 h) semi-static renewal system in 60 litre glass aquaria filled with tap water (for *Marisa*) or artificial seawater (for *Nucella*; salinity 35‰) and provided with an Eheim power filter was used. The tests were performed under constant conditions with a temperature of 22 ± 1°C for freshwater and 14 ± 1°C for marine snails; the light dark cycle was adjusted to 12:12 h.

Three different series of exposure experiments were conducted with the test compounds bisphenol A (BPA, Merck Schuchardt, Germany) and octylphenol (OP, Merck Schuchardt, Germany):

1. ***Marisa* P (parental generation)-test:** Adult *Marisa cornuarietis* of comparable age were exposed to nominal concentrations of 1, 5, 25, and 100 μg BPA or OP/L for 5 months, including a solvent control (ethanol). Thirty specimens from each group were collected for analysis at the beginning of the experiment and at monthly intervals.
2. ***Marisa* LC (life-cycle)-test:** The spawning masses with eggs produced by the adult ramshorn snails in the solvent control and in the 1 and 100 μg BPA or OP/L groups during the *Marisa* P-test were further exposed to these nominal concentrations over a period of 12 months until the hatched F_1 specimens were one year old. They reached sexual maturity in

their 8th month. Thirty specimens from each group were collected for analysis at an age of 6, 8, and 12 months. Additionally, the hatching success of the F_1 generation was recorded.

3. ***Nucella* test:** Adult *Nucella lapillus* were exposed to nominal concentrations of 1, 25, and 100 μg BPA or OP/L for 3 months, including a solvent control (glacial acetic acid). Thirty specimens from each group were collected for analysis at the beginning of the experiment and at monthly intervals.

During the experiments mortality and production of spawning masses with the number of eggs in each of the aquaria were recorded at daily intervals. As the fecundity parameters could not be assessed for individual females but only for the single experimental groups in a tank with a known number of females following the analyses of specimens, no measures of variability (e.g. standard deviation or standard error) can be calculated for these endpoints.

All specimens were narcotised prior to analysis (2.5% $MgCl_2$ in distilled water for *Marisa*, 7% $MgCl_2$ for *Nucella*). The individual shell and aperture height were measured to the nearest 0.1 mm before the shell was cracked and the snail was removed. The presence, normal appearance, and extension (to the nearest 0.1 mm) of all sex organs was checked as well as the occurrence of oocytes and sperm in the genital system and of visible excrescences on genital and other organs with a dissection microscope. Additionally, the VDSI (vas deferens sequence index = mean value of imposex stages in a sample with values of 0 to 3 in *Marisa cornuarietis* and 0 to 6 in *Nucella lapillus*) as a measure of the imposex intensity in a sample was calculated (for details see Oehlmann *et al*., 1991; Schulte-Oehlmann *et al*., 1995).

For histopathological analyses of the gonads during the *Marisa* P-test, 6 male and 6 female specimens from each sample were fixed in Carnoy's and Bouin's fluid, respectively, and then preserved in ethanol. After embedding in paraplast, serial sections (5–7 μm) were made and stained with haemalun-chromotrope. The sections were analysed using an image analysis system (Optimas 5.2, Optimas Cooperation) coupled with an Olympus microscope (BX 50).

Standard statistical analyses of the results (e.g. analyses of covariance (ANCOVA) with multiple comparison of samples according to Tukey (low n) or Student-Newman-Keuls (high n), H test (Kruskal-Wallis test with multiple comparison of samples according to Nemenyi), χ^2 test, and Weir test for classified values) were performed according to Weber (1972) and Lozán (1992) using the computer programme StatEasy for Windows NT.

Results and discussion

Marisa P (parental generation)-test

During the first series of laboratory experiments with adult *Marisa cornuarietis* a complex syndrome of morphological and physiological alterations occurred, resulting in an enhancement of spawning mass and egg production, malformations of the female genital system, and probably also in a higher female mortality. We refer to this syndrome as the "induction of superfemales." Superfemales are female specimens with additional female sex organs, e.g. a second vagina with vaginal opening to the mantle cavity (Fig. 1) and/or an enlargement of the pallial accessory sex glands (albumen and capsule gland, Figs. 2a,b).

The normal morphological and histological structure of the male and female genital system of the ramshorn snail and of pathomorphological changes during imposex development following a TBT exposure is documented by Schulte-Oehlmann *et al*. (1994, 1995). Superfemales were not observed before in our laboratory although more than 8,000 specimens have been analysed since 1992. Additional female sex organs, like in the ramshorn snail specimen documented in Fig. 1, occurred only exceptionally as individual cases during the experiments, but the albumen and capsule glands were enlarged in all females in the BPA and OP experimental groups irrespective of the concentration of the xeno-estrogens. The direct comparison of the pallial gland complex of a typical female from the control group (Fig. 2a) with a specimen in a xeno-estrogen treated group (Fig. 2b) shows that primarily the volume of the capsule and albumen gland increased but the maximum extension or length of these organs,

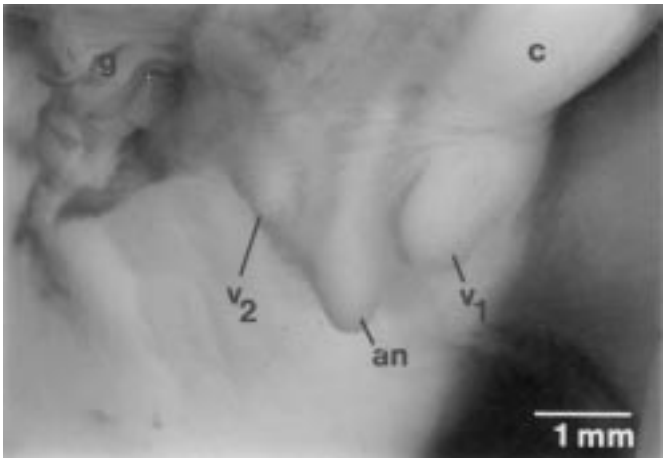

Figure 1. *Marisa cornuarietis*. Photograph of a superfemale with an additional vagina including female opening. Abbreviations: an, anus; c, capsule gland; g, gill; v_1, original vagina in normal position; v_2, additional vagina on other side of the rectum.

which was measured during the experiments, was more or less unaffected (no statistically significant differences in extension; H test, $p > 0.05$).

This hypertrophy of the two female glands was not the ultimate effect of the superfemale syndrome. In a number of the BPA- and OP-treated females a rupture of the pallial oviduct occurred as shown in Fig. 2c, irrespective of the applied concentration. The incidence of this phenomenon was 3.73% three months after start of the experiment. The opening was typically found at the transition zone between the albumen and capsule gland and in some of these superfemales a protrusion of spawning mass (egg capsules filled with eggs and intracapsular fluid) was found at the rupture (Fig. 2d).

Additionally, in all experimental groups exposed to BPA and OP some females with abortive capsule masses in the lumen of the albumen gland were observed (Fig. 3). These capsules accumulated in the tubular part of the gland which continues in the capsule gland. Therefore, the abortive mass was found in the same region where the rupture of the oviduct occurs.

The protrusion of spawning masses and the accumulation of abortive egg capsules in the oviduct gave an indication for the underlying causes for the enlargement of the female pallial glands and the rupture of the oviducts. It was the enhancement of the spawning mass and egg production in all BPA- and OP-treated groups compared to the control during the experiments, counted by successful egg laying. This is not only true if the cumulative numbers were recorded during the experiments (Fig. 4) but also when the corresponding values per female were analysed (Fig. 5). An ANCOVA analysis with multiple comparison of samples according to Student-Newman-Keuls reveals that the spawning mass and egg production in all BPA- and OP-treated groups was significantly higher than in the control group ($p < 0.01$). For the OP experiment, an inverted U-type concentration response relationship exists with the highest concentration (100 μg OP/L) evoking comparable effects like the second lowest (5 μg OP/L) (no significant differences in Student-Newman-Keuls test). The lower effectiveness of the highest concentration of 100 μg/L compared to the second highest (25 μg/L) was much less pronounced in the BPA test series giving only little evidence for a comparable inverted U-type concentration response relationship as for OP.

The detailed investigations of TBT effects demonstrated in a number of muricid gastropod species (e.g. *Nucella lapillus*, *Ocinebrina aciculata*) a comparable rupture of the pallial oviduct as observed in BPA and OP exposed ramshorn snails due to a blockade of the vaginal opening by proliferating vas deferens tissue and a resulting accumulation of abortive capsule masses. In such affected populations an increased female mortal-

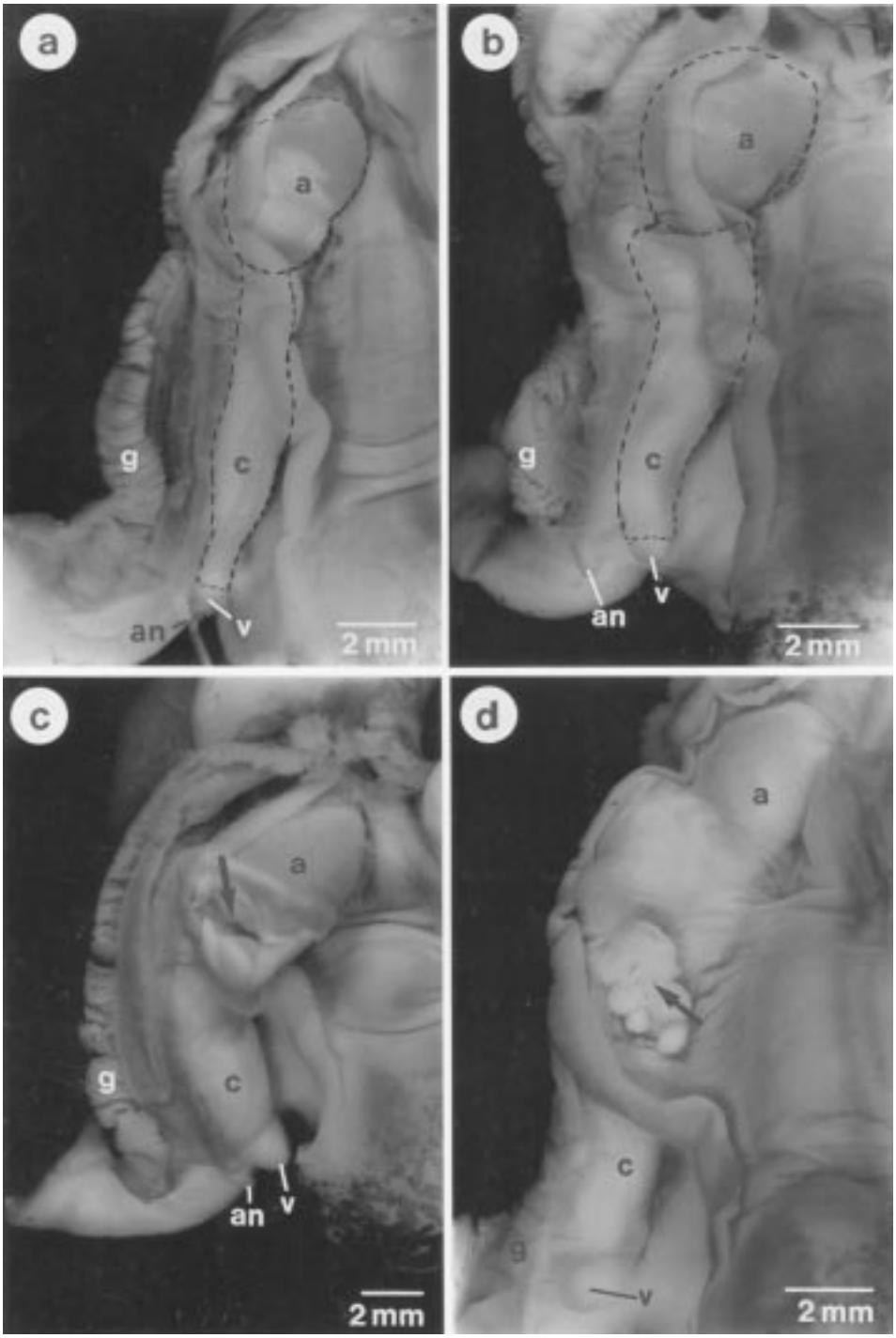

Figure 2. Marisa cornuarietis. Photographs of a control female (a) and of BPA or OP treated superfemales (b–c) with opened mantle cavities. In (b) the enlargement of the capsule and albumen gland is indicated by the broken line in comparison to the control female in (a). In (c) a rupture in the wall of the pallial oviduct occurs (arrow) and in (d) additionally a protrusion of the spawning mass is visible at the rupture (arrow). Abbreviations: a, albumen gland; an, anus; c, capsule gland; g, gill; v, vagina.

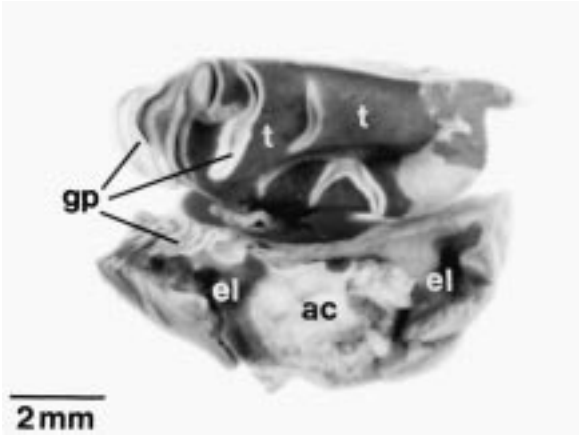

Figure 3. Marisa cornuarietis. Photographs of an albumen gland from a control animal (above) and a BPA treated female (below) with an abortive capsule mass opened by a longitudinal section. Abbreviations: ac, abortive capsule mass; el, extension of albumen gland lumen; gp, glandular pouches of the albumen gland; t, tubular part of the albumen gland.

ity and a consequent shift of the sex ratio in favour of males was observed (Gibbs *et al*., 1987; Oehlmann *et al*., 1996). Therefore, it can be assumed that not all *Marisa* superfemales with an oviduct rupture were found at the monthly analyses of the samples with thirty specimens per group as it is likely that these females will not survive for long after the rupture occurred. The mortality data presented for the *Marisa* P-test in Fig. 6 show clearly a statistically significant increase in all BPA and OP exposed groups of snails compared to the control. Because the two test compounds exhibit in general a very low acute toxicity (BUA, 1991, 1997), the elevated mortality seems not to be a direct effect of BPA and OP, but a result of the induction of superfemales with a rupture in the oviduct. It was not possible to sex the dead specimens due to their rapid decay but in all experimental groups, a shift of the sex ratio in favour of males was detectable when compared to the control although these differences were not statistically significant (χ^2 test, $p > 0.05$).

The histopathological analyses of the gonads of both sexes during the *Marisa* P-test gave no indication that spermiogenesis or oogenesis were affected by either BPA or OP in the applied concentration range.

Marisa LC (life-cycle)-test

The hatching success of the F_1 generation during the *Marisa* LC-test was not affected by the two test compounds. In general, BPA and OP produced comparable effects in the F_1 generation as already demonstrated for the P-test above. Only slight differences regarding a higher incidence of sterilised superfemales due to a rupture of the oviduct and a less striking enhancement of spawning mass and egg production at the highest concentration were observed (Fig. 7).

The first specimens with oviduct ruptures were already found when the first females reached sexual maturity at an age of 6 months in the two tested OP concentrations (1 and 100 μg/L). The incidence was 5.0% for the lower and 9.1% for the higher concentration (mean for both OP treatments: 6.5%; mean for BPA: 0%). At an age of eight months the percentage of females with these oviduct malformations increased to 15.4% in the 100 μg OP/L group (mean: 7.7% for OP and 0% for BPA). At the end of the experiment, when all females were sexually mature, superfemales with oviduct ruptures were also found for the first time in the two BPA exposure groups during the *Marisa* LC-test. The incidences are 5.2% for the lower and 11.8% for the higher BPA (mean: 8.3%), 7.4% for the lower and 10.0% for the higher OP concentration (mean: 8.1%). Although more sterilised superfemales than in the P-test were found during the *Marisa* LC-test and these specimens did only occur in the xeno-estrogen treated groups in both experiment series, the differences were not statistically significant (χ^2 test; $p > 0.05$). As during the P-test, the mortality was also statistically significantly higher in all xeno-estrogen exposed cohorts of the LC-test (range: 14.8–21.5%) when compared to the control (8.6%) between month 6 and the end of the experiment (χ^2 test; $p < 0.05$).

As already stated, the enhancement of the spawning mass and egg production was less marked for the F_1 generation in the highest concentration of BPA and OP although statistically significant when compared to the control (ANCOVA analysis with multiple comparison of samples according to Tukey; $p < 0.05$). The differences between the two xeno-estrogen concentrations were not significant. The reduced effect of

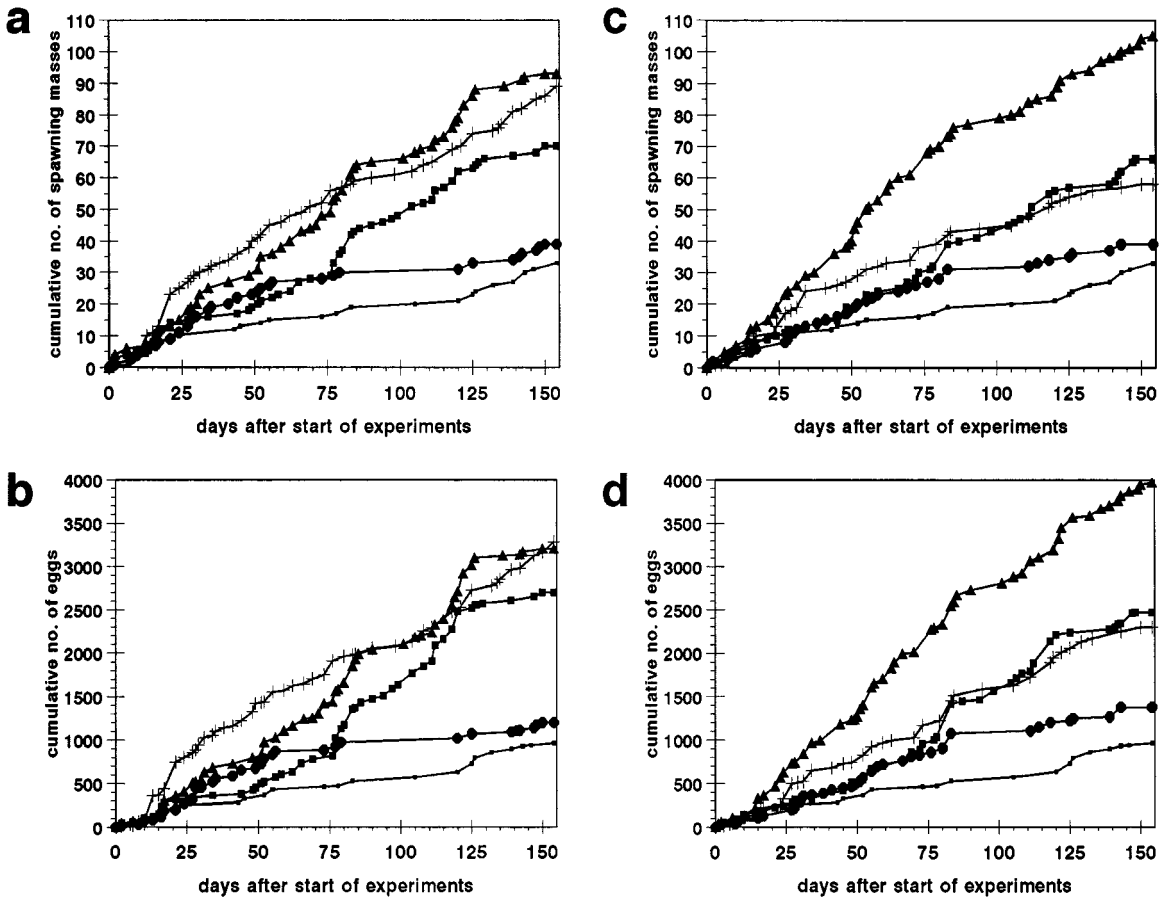

Figure 4. Marisa cornuarietis. Cumulative numbers of spawning masses (a, c) and eggs (b, d) produced by all females of the single experimental groups exposed to BPA (a, b) and OP (c, d) during the *Marisa* P-test. Exposure groups: (·) solvent control, (●) 1 μg/L, (■) 5 μg/L, (▲) 25 μg/L, (+) 100 μg/L.

100 μg BPA or OP/L in the F_1 can be interpreted as a loss of sensitivity which might be due to a down-regulation of the estrogen receptors. It has been described for a number of vertebrate species that a long term application of estrogen agonists resulted in a down-regulation of estrogen receptors (reduced numbers and/or sensitivity) and simultaneously in an up-regulation of androgen receptors (higher numbers and/or sensitivity) (Marquardt and Schäfer, 1994).

At the end of the *Marisa* LC-test, when all females were sexually mature, the imposex intensities increased in the 100 μg/L exposure groups of BPA and OP (Fig. 8). This virilisation was only significant for BPA (Weir test for classified values, $p < 0.001$) and was not observed during the P-test with adult ramshorn snails. The above mentioned up-regulation of androgen receptors as a result of a long term exposure to high xenoestrogen concentrations offers a possible explanation for the enhanced VDSI values. The unchanged endogenous testosterone titres are supposed to have this effect as the androgen can bind to a higher number or more sensitive androgen receptors in the tissue.

Nucella test

Also during the three months experiment series with the dogwhelk *Nucella lapillus* an induction of superfemales was observed with an increase of oocyte production (Fig. 9) and a hypertrophy of the pallial female sex glands (Fig. 10). In contrast to *Marisa*, no gross malformations of the

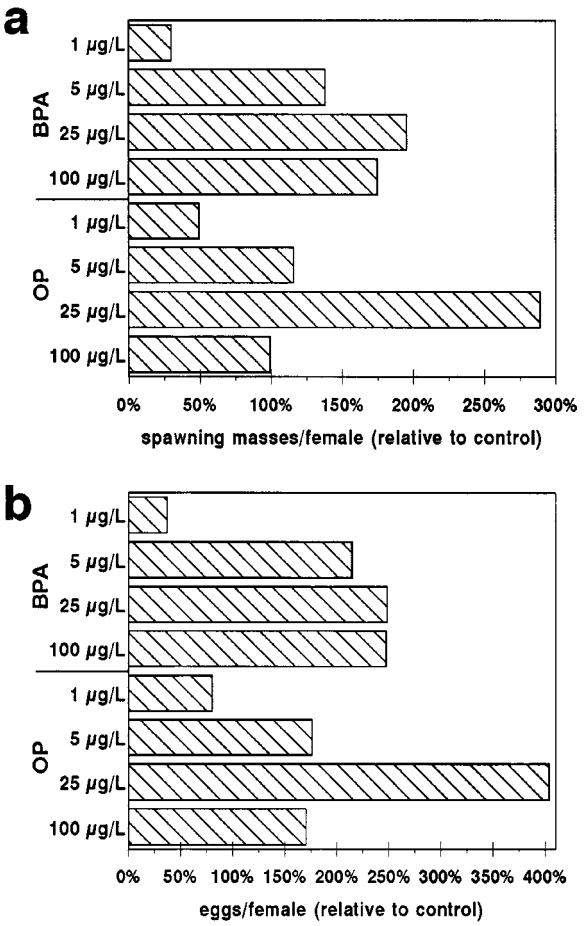

Figure 5. Marisa cornuarietis. Comparison of spawning mass (a) and egg numbers per female (b) relative to the control group produced during the *Marisa* P-test. As both parameters could not be assessed for individual females but only for the single experimental groups (with a known number of females) the bars are calculated on the basis of mean values. Consequently, it is not possible to present a measure of variability within a group.

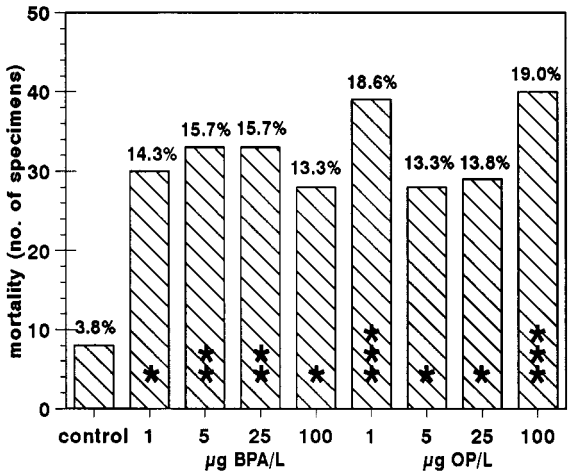

Figure 6. Marisa cornuarietis. Mortality (in relative and absolute values) in the experimental groups during the *Marisa* P-test. Asterisks indicate statistical significant differences to control (χ^2 test): ★, $p < 0.05$; ★★, $p < 0.01$; ★★★, $p < 0.005$.

oviduct occurred like the slit-like ruptures at the anterior region of the albumen gland as described above and consequently also no female sterilisation nor a higher overall or female mortality. The main reason for this is the morphological difference in the structure of the pallial oviduct section in the ramshorn snail and in dogwhelks which do not exhibit a comparable bottleneck passage for the sexual products in the proximal section of the oviduct (Oehlmann *et al.*, 1988; Schulte-Oehlmann *et al.*, 1994). But beyond this it has also to be taken into account that *Nucella lapillus* does normally not produce egg capsules when the specimens are transferred from the field into the laboratory. Under these constant conditions, the produced oocytes are digested in the ingestion gland. In the field, the raise of oocyte production demonstrated in Fig. 9 will result in a consequent stimulation of egg capsule formation. If capsule production in dogwhelks is comparably excessive increased as in *Marisa* by xeno-estrogens, the sexual products might congest in the distal part of the capsule gland leading to comparable individual and population effects as in the final stages of imposex development when females are sterilised due to a blockade of the oviduct by proliferating vas deferens tissue: accumulation of abortive capsule masses, distension and rupture of the pallial oviduct, and consequently an increased female mortality.

The accessory female pallial sex glands in *Nucella lapillus* (albumen, ingestion and capsule gland) were enlarged in all BPA and OP groups compared to the control already after the first and second month of exposure although these differences were not statistically significant. As already stated for *Marisa*, primarily the volume of the capsule and albumen gland appeared to be increased but the maximum extension of these organs, which was exclusively measured at these time points, was more or less unaffected. There-

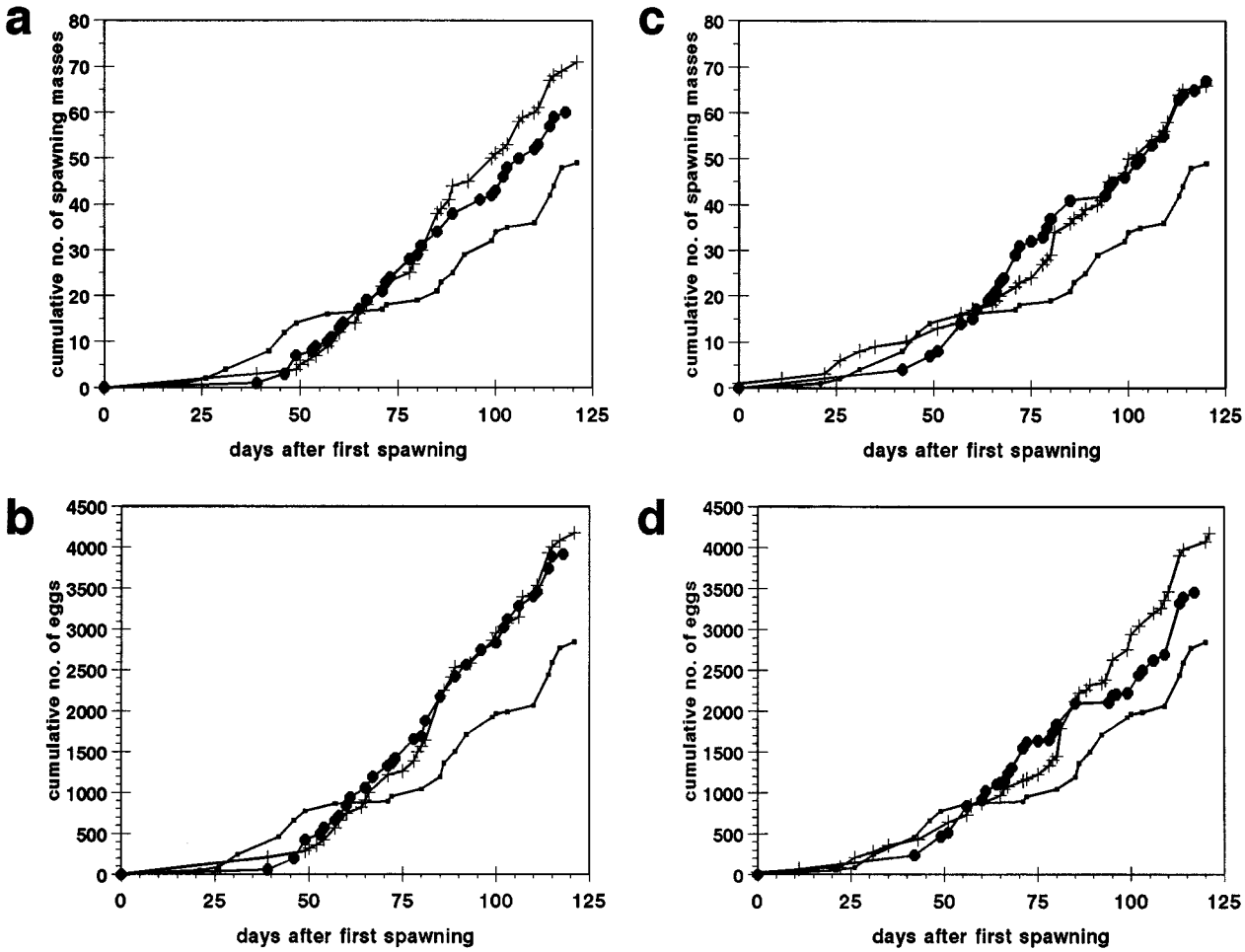

Figure 7. *Marisa cornuarietis*. Cumulative numbers of spawning masses (a, c) and eggs (b, d) produced by the experimental groups exposed to BPA (a, b) and OP (c, d) during the *Marisa* LC-test (F_1 generation). Exposure groups: (·) solvent control, (●) 1 μg/L, (+) 100 μg/L.

fore, at the end of the 3 months experiment not only the extension of the three pallial glands was measured (Fig. 10a with results for the capsule gland) but also the weight of the entire complex (Fig. 10b) with significant differences between all groups which received the two xeno-estrogens and the control (Kruskal-Wallis test with multiple comparison of samples according to Nemenyi; $p < 0.001$). It has to be considered that the test animals were sampled during the breeding season in March and the test was started in April when the female glands still attain almost their maximum natural size (Oehlmann, 1994). The effects of the two test compounds on the pallial oviduct mass would be probably more obvious at another time of year when the female gland complex is smaller.

The increase in weight of the female pallial gland complex under the influence of xeno-estrogens is a very easily measurable endpoint which opens the possibility to use prosobranch snails in a comparable manner as ovariectomised rodents for a kind of invertebrate uterotrophic assay.

Contrary to the findings for *Marisa cornuarietis*, the males were affected by the two test compounds in *Nucella lapillus*. Already after the first month of exposure, the percentage of males with sperm stored in the vesicula seminalis dropped to 64.7–75.0% in the BPA and 63.6–

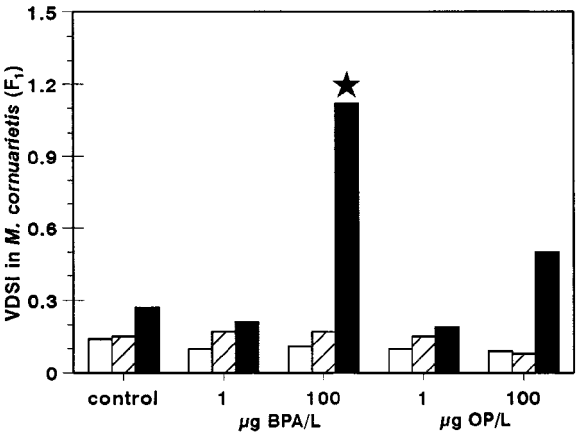

Figure 8. Marisa cornuarietis. Imposex intensities, measured as the vas deferens sequence index (VDSI), during the *Marisa* LC-test (F_1 generation) at an age of 6 (white bars), 8 (hatched bars) and 12 months (black bars). The asterisk indicates a statistical significant difference to control (Weir test for classified values, $p < 0.001$).

88.9% in the OP-treated groups while the corresponding value was 100% for the control males. These differences were statistically significant (χ^2 test; $p < 0.05$) with the exception of the 1 μg OP/L group (88.9%). At the end of the second and third month, none of the xeno-estrogen exposed males had sperm in this storage organ but

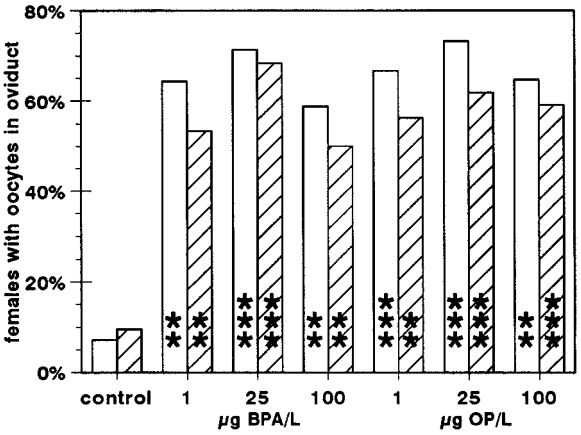

Figure 9. Nucella lapillus. Relative numbers of females with oocytes in the oviduct during the *Nucella* test with BPA and OP after 2 (white bars) and 3 months (hatched bars) of exposure. Asterisks indicate statistical significant differences to control (χ^2 test): ★★, $p < 0.01$, ★★★, $p < 0.001$. The differences for the first month sample were not statistically different (χ^2 test, $p > 0.05$).

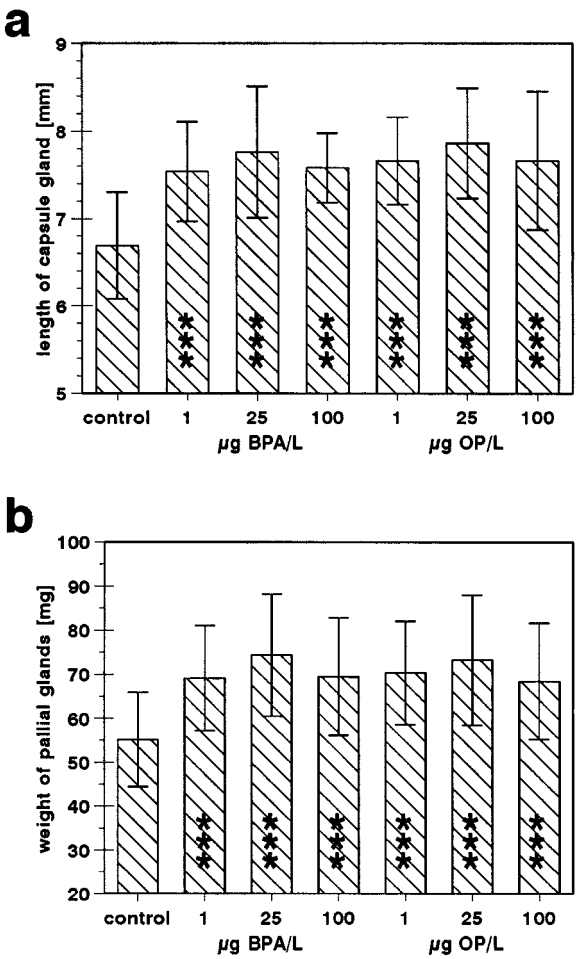

Figure 10. Nucella lapillus. Comparison of the length of the capsule gland (a) and the weight of the female pallial glands (b) at the end of the *Nucella* test (3 months of exposure). Mean values and standard deviation are shown (sample sizes: control: 21; 1 μg BPA/L: 15; 25 μg BPA/L: 19; 100 μg BPA/L: 20; 1 μg OP/L: 16; 25 μg OP/L: 21; 100 μg OP/L: 22 females). Asterisks indicate statistical significant differences to control (H test (Kruskal-Wallis test)): ★★★, $p < 0.001$.

still 18.8% (month 2) or 12.5% (month 3) in the control. This finding is an indication that BPA and OP might advance sexual repose at the end of the breeding seasons.

Additionally, the length of the male penis and of the prostate gland were significantly reduced when compared to the control (ANOVA with multiple comparison of samples according to Student-Newman-Keuls, $p < 0.05$). In Figure 11 the results after three months are presented but this

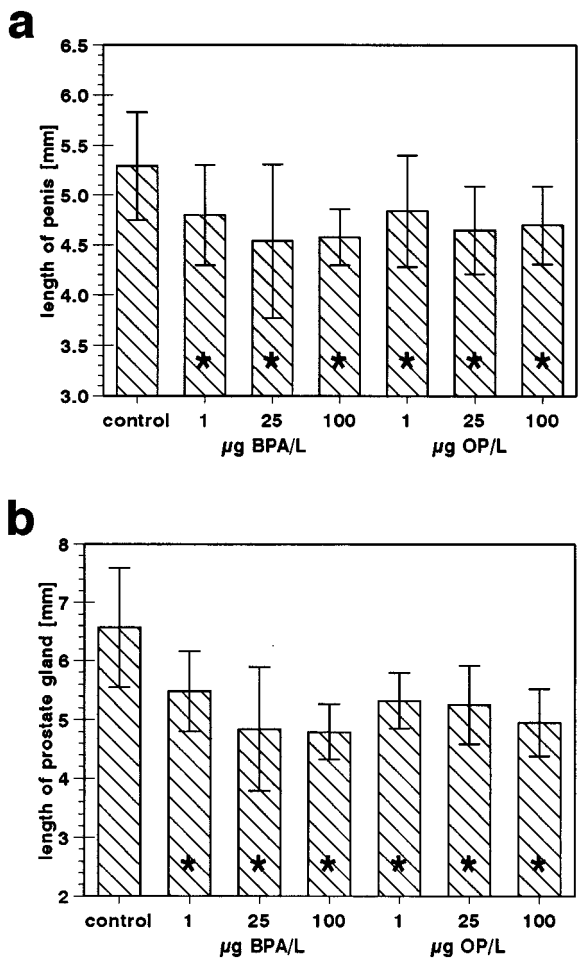

Figure 11. Nucella lapillus. Comparison of the penis (a) and prostate gland length (b) at the end of the first exposure 3 month. Mean values and standard deviation are shown (sample sizes: control: 16; 1 µg BPA/L: 12; 25 µg BPA/L: 17; 100 µg BPA/L: 12; 1 µg OP/L: 18; 25 µg OP/L: 15; 100 µg OP/L: 11 males). Asterisks indicate statistical significant differences to control (ANOVA with multiple comparison of samples according to Student-Newman-Keuls): ★, $p < 0.05$.

effect was observable already four and eight weeks after the start of the experiment. During copulation, the male penis is inserted into the bursa copulatrix of the female which is positioned in the distal section of the pallial oviduct. Therefore, a minimum extension of the penis is necessary for a successful transfer of sperm. The reduced penis and prostate gland length as well as possible advancement of sexual repose might have negative implications for the reproductive success of males also in the field.

A comparison of our own results with other reports in the literature is difficult because only a few studies have investigated the hormone-mimetic effects of BPA, OP or related xeno-estrogens in invertebrates so far. The results for the standard invertebrate test organism in aquatic ecotoxicology, the water flea *Daphnia magna* are conflicting. Zou and Fingerman (1997) found a reduced moulting frequency in their experiments with xeno-estrogens but these findings could not be confirmed by Caspers (1998) for BPA. He criticises the suitability of this endpoint for the assessment of endocrine-mimetic properties of test compounds as it is at least doubtful whether or not steroids have a functional role in crustaceans (deFur *et al.*, 1999).

Our findings are supported by the investigations of Andersen *et al.* (1999b) who revealed an increase of egg production if the copepod *Acartia tonsa* was exposed to either 23 µg 17ß-estradiol/L or 20 µg BPA/L. In fathead minnows (*Pimephales promelas*) low aqueous concentrations of 0.05 µg 4-nonylphenol/L also resulted in an enhancement of egg production (Giesy *et al.*, 2000). These authors observed a comparable inverted U-type concentration response relationship as it was found for the endpoints spawning mass and egg production in our OP experiments with *Marisa*.

Zou and Fingerman (1999) found a reduced chitobiase activity in the fiddler crab, *Uca pugilator*, when exposed to 10 mg OP/L for 7 days. Although the reduced chitobiase activity might result in a slowing of moulting it seems at least questionable whether this effect is endocrine-mediated.

In contrast to the small number of studies on xeno-estrogen effects on invertebrates, there are numerous publications investigating the responses of BPA and OP in in-vitro tests or tests with vertebrates, but partially with conflicting results. Andersen *et al.* (1999a) analysed the estrogenicity of 20 chemicals in 8 different short-term assays. While BPA induced an estrogenic response in all assays, the results for OP varied among assays in the single laboratories indicating that additional standardisation of the test protocols is required. Lutz and Kloas (1999) report a comparable binding affinity of both compounds to the estrogen receptor of the amphibian *Xenopus*

laevis. If *X. laevis* was exposed to low concentrations of either BPA or OP during larval development, a significantly higher number of female phenotypes occurred in the adults compared to controls (Kloas *et al*., 1999).

vom Saal *et al*. (1998) stated that OP and BPA reduce the daily sperm production in male offspring of mice fed these chemicals from gestation day 11 to 17 at doses as low as 2 μg/kg body weight. These results could not be confirmed by Ashby *et al*. (1999). Additionally, Takao *et al*. (1999) found that a prepubertal and pubertal oral administration of BPA resulted in a decrease of plasma free testosterone levels and alterations in the differentiation of the male reproductive tract. Howdeshell *et al*. (1999) report an increased postnatal body weight gain and an advancement of puberty in female CF-1 mice whose mothers were treated with 2.4 μg BPA/kg on days 11 to 17 of gestation.

Laws *et al*. (2000) tested the estrogenic activity of BPA and OP in comparison to other xenoestrogens, ethinylestradiol, and 17ß-estradiol in feeding experiments with prepubertal and ovariectomised adult rats (3-day uterotrophic assay). They showed that both compounds were estrogenic at daily oral doses of 50–200 mg/kg (OP) and 100–200 mg/kg (BPA). Sharpe *et al*. (1995) report a reduced testicular size and sperm production in male offspring of Wistar rats which were exposed to low doses of BPA during gestation and lactation via the drinking water but these results could not be confirmed by Cagen *et al*. (1999).

Although the relevance of all reported effects of BPA and OP in vertebrates was not checked for *Marisa cornuarietis* and *Nucella lapillus*, either because the experimental design was unsuited for this purpose (e.g. advancement of puberty; modulation of mating behaviour) or the samples are not yet analysed (e.g. spermiogenesis and oogenesis in F_1 specimens), it is obvious that a number of responses are comparable. This is true especially for the stimulation of egg production and the positive uterotrophic effect in both species as well as for the reduction of penis and prostate gland length in male *Nucella*. Another important point is that both snail species exhibit already marked effects at the lowest nominal test concentrations indicating that LOEC (lowest observed effect concentration) values might be still lower. Under certain circumstances, like in ramshorn snails due to their specific morphological structure of the pallial oviduct, these alterations can be expected to exhibit serious consequences at the population level. The occurrence of superfemales with a rupture in their oviduct is a very striking parallel case to the deleterious effects of tributyltin compounds on muricid gastropods in many coastal regions of the world. The mechanistical studies of imposex and intersex induction in prosobranchs showed clearly that steroids play an important role in this group of invertebrates and that even some of the basic regulatory mechanisms known for vertebrates are present (Bettin *et al*., 1996; Matthiessen and Gibbs, 1998). We hope that the results communicated in this paper and in the next publications dedicated to xenoandrogens and antiandrogens will help to demonstrate that invertebrates, like vertebrates, are sensitive to endocrine disruption at environmentally relevant concentrations and additionally that they are provided with complex feed back mechanisms in their steroid metabolism.

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