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Chapter 19

Effects of Ethinylloestradiol and Methyltestosterone in Prosobranch Snails

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19.1 Introduction

Recent reports have shown that a number of pharmaceuticals do occur not only in raw sewage but also in effluents of sewage treatment works, sewage sludge and receiving surface waters (Daughton and Ternes 1999; Kümmerer 2001). The list of pharmaceuticals detected in aquatic ecosystems is steadily increasing while almost nothing is known regarding their potential effects on aquatic wildlife. Although concentrations of pharmaceuticals in the aquatic environment are generally in the lower ng l⁻¹ and µg l⁻¹ range, it has to be considered that these compounds were developed to exhibit a high biological activity, often associated with a high stability so that they are not readily biodegradable. Therefore, concerns have been raised regarding the potential impact from such compounds on aquatic wildlife even at the low reported environmental concentrations because of unknown safety factors and because accumulating compounds may attain much higher concentrations in organisms than in the water phase.

In contrast to the limited ecotoxicological information which is available for most of the pharmaceuticals being detected in aquatic ecosystems, the case of the synthetic steroid 17 α -ethinylloestradiol, used in oral contraceptives, provides an example that wildlife fish populations may be affected by pharmaceuticals even at the sub-ng l⁻¹ range (Young et al. 2002). Almost nothing is known for other pharmaceuticals in this respect. Furthermore, the majority of newly started research in this field is dedicated to the effects of pharmaceuticals on aquatic vertebrates, namely fish. The potential impact on invertebrates is by far less considered although it has been shown that the same or comparable substances like in vertebrates may interfere with hormonal processes in invertebrates, affecting developmental processes, growth and reproduction and thus threatening the survival of wildlife populations (for a review: Oehlmann and Schulte-Oehlmann 2003). Studies on potential effects of pharmaceuticals in invertebrates are important because invertebrates represent not only more than 95% of the known species in the animal kingdom, but provide also key species for ecosystem functioning and represent an even today not sufficiently characterized although extremely important part of the global biodiversity.

In general, endocrine systems of invertebrates have not been as well documented as those of vertebrates, nor have responses of invertebrate endocrine systems to suspected endocrine disrupting chemicals (EDCs) been studied with comparable intensity. On the other hand, there is now considerable evidence that prosobranch snails are affected by the same EDCs exhibiting either an oestrogenic (Oehlmann et al. 2000; Duft et al. 2003b) or androgenic activity (Schulte-Oehlmann et al. 2000; Duft et al. 2003a).

It was the objective of the present study to investigate the effects of the synthetic steroids 17 α -ethinyloestradiol (EE₂) and 17 α -methyltestosterone (MT) in the freshwater ramshorn snail *Marisa cornuarietis* (Mollusca: Gastropoda: Prosobranchia). These experiments were part of a broad test program analyzing the effects of industrial chemicals and pesticides, suspected as endocrine disrupters, in a range of different snail species. During these experiments endocrine active pharmaceuticals such as the potent receptor agonists EE₂ and MT, specific aromatase inhibitors, competitive anti-androgens and anti-oestrogens were used as positive controls.

19.2 Materials and Methods

The experiments were performed with the gonochoristic prosobranch snail, *Marisa cornuarietis*. 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. For all experiments, a 24 h (weekends: 48 h) semi-static renewal system in 60 liter glass aquaria filled with conditioned, active charcoal-filtered tap water and provided with an Eheim power filter was used. The tests were performed under constant conditions with a temperature of 22 \pm 1°C with the light dark cycle being adjusted to 12:12 h.

Three different series of exposure experiments were conducted with the test compounds 17 α -ethinyloestradiol (EE₂, CAS No. 57-63-6, Fluka Chemie AG, Article No. 02463) and 17 α -methyltestosterone (MT, CAS No. 58-18-4, Sigma Chemicals, Article No. M7252):

1. Adult exposure experiment (**AE**): Sexually mature *M. cornuarietis* of comparable age and size were exposed to nominal concentrations of 0.1; 0.25; 0.5 and 1 μ g EE₂ l⁻¹ or MT l⁻¹ for 6 months, including a solvent control (ethanol). Thirty specimens from each group were collected for analysis at the beginning of the experiment, 1, 2 and 4 weeks after start of the test and consequently at monthly intervals.
2. Juvenile exposure experiment (**JE**): Sexually immature *M. cornuarietis* of comparable age and size were exposed to the same nominal concentrations as described for the AE series above for 6 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.
3. Low concentration experiment with EE₂ (**LC**): Sexually mature *M. cornuarietis* of comparable age and size were exposed to nominal concentrations of 1; 10; 25; 50, and 100 ng EE₂ l⁻¹ for 6 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.

The different parameters for the assessment of mortality, reproductive output and morphological alterations in the test specimens are described in detail in Oehlmann et al. (2000) and Schulte-Oehlmann et al. (2000), including histopathological analyses and the statistical evaluation of test results. As one of the most important endpoints considered here, the VDSI (vas deferens sequence index = mean value of imposex stages in a sample with values of 0 to 3 according to Fig. 19.1) as a measure of the imposex intensity in a sample was calculated (cf. Schulte-Oehlmann et al. 1995).

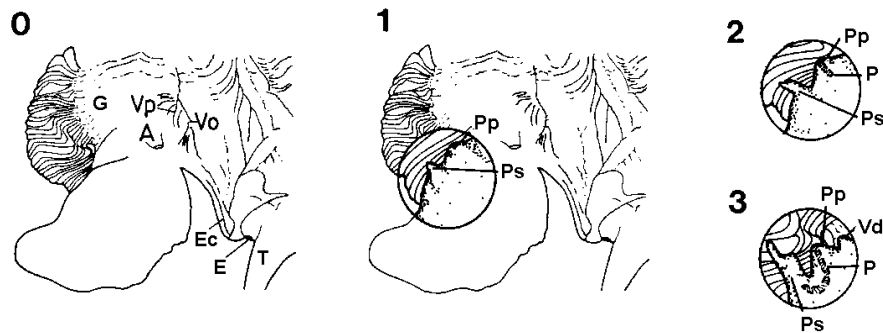


Fig. 19.1. *Marisa cornuarietis*. Females with their mantle cavity opened to demonstrate the occurrence and extension of male organs for stages 0 – 3 of imposex development. A, anus; E, eye; Ec, egg channel; G, gill; P, penis; Pp, penis pouch; Ps, penis sheath; T, tentacle; Vd, vas deferens; Vo, vaginal opening; Vp, vaginal papilla

19.3

Results and Discussion

19.3.1

Effects on Females at Concentrations from 0.1 to 1 $\mu\text{g l}^{-1}$

One of the most drastic effects of EE₂ and MT in prosobranch snails in the tested concentration range is a time-dependent induction of imposex. For the ramshorn snail *Marisa cornuarietis* three imposex stages additionally to the unaffected female (stage 0, Fig. 19.2a) can be distinguished (Fig. 19.1). Stage 1 represents the level of imposex, female *M. cornuarietis* may already exhibit naturally (Schulte-Oehlmann et al. 1995). Two small swellings on the top of the inner side of the mantle cavity represent the oriments of penis sheath and penis pouch. The imposex stages 2 and 3 are only found in snails exposed to endocrine active substances. Both, penis sheath and pouch grow gradually and a little penis appears as a new formation in stage 2. Additionally, stage 3 is characterized by a vas deferens and enlarged male sex organs (Fig. 19.2b).

The effects of MT and EE₂ on imposex induction are summarized in Fig. 19.3 for the adult exposure experiment (AE). Regarding its virilization capabilities, MT is obviously more effective than EE₂. Females exposed to MT develop imposex earlier and attain higher VDSI values compared to those exposed to EE₂. Already after four weeks VDSI values have increased in MT exposed groups but only for the highest concentration the effect is statistically significant ($p < 0.05$, Weir test; Fig. 19.3b). From month 3 on VDSI values are statistically significantly higher in all MT exposed groups when compared to the control ($p < 0.05$, Weir test).

The lowest applied EE₂ concentration of 0.1 $\mu\text{g l}^{-1}$ during the AE experiment did not show any statistically significant imposex promoting effect even after an exposure of 6 months, while an exposure to concentrations between 0.25 and 1.0 $\mu\text{g EE}_2 \text{ l}^{-1}$ resulted in a statistically significant VDSI increase from month 4 on ($p < 0.05$, Weir test; Fig. 19.3a). At the end of the experiment, imposex intensities in the EE₂ exposed groups turned out to be concentration dependent, while the response in snails exposed to MT

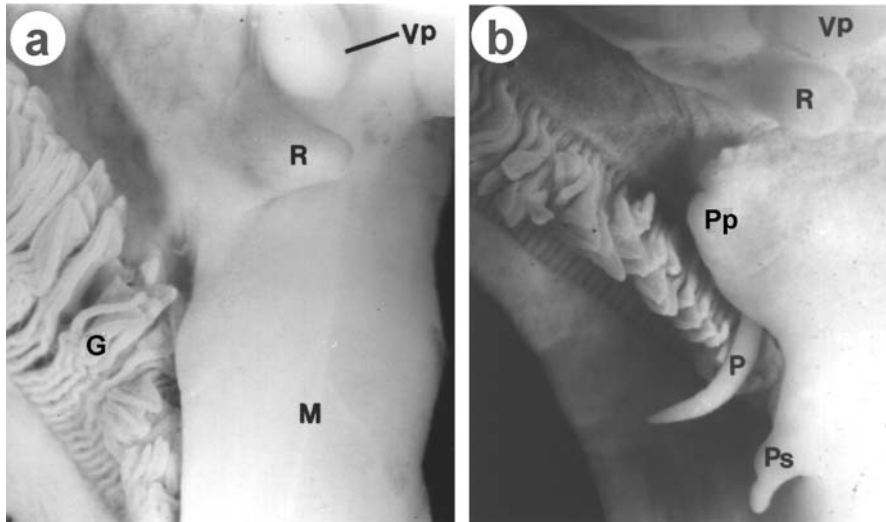
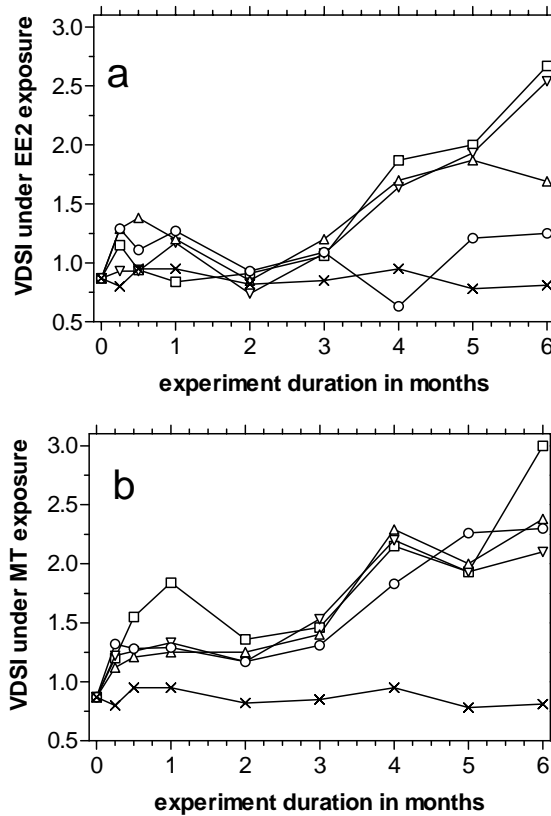


Fig. 19.2. *Marisa cornuarietis*. Photographs of the mantle cavity of females; **a** without imposex (Stage 0) from the control group; **b** of imposex stage 3 exposed to $1 \mu\text{g MT l}^{-1}$. G, gill; M, mantle epithelium; P, penis; Pp, penis pouch; Ps, penis sheath; R, rectum; Vp, vaginal papilla

was almost independent from the concentration applied with $0.1 \mu\text{g MT l}^{-1}$ provoking a comparable virilization of females as $1.0 \mu\text{g l}^{-1}$. The potency of MT in *M. cornuarietis* is demonstrated by the fact that all females attained the ultimate degree of imposex development (stage 3) at the end of the experiment in the group receiving $1.0 \mu\text{g MT l}^{-1}$. The results from the AE experiment were confirmed for sexually immature snails in the identical EE_2 and MT concentration range (JE experiment series; data not shown). But in contrast to our expectations and literature data indicating a higher susceptibility of younger specimens (e.g. Mensink et al. 1996), juvenile snails did not show a higher sensitivity compared to sexually mature adults.

The finding of an oestrogenic compound such as EE_2 being able to induce a virilization of females seems to be inconsistent with its specific mode of action at first glance. It has been shown in experiments conducted by Schulte-Oehlmann et al. (2001a) that a simultaneous administration of EE_2 at concentrations from 0.1 to $1.0 \mu\text{g l}^{-1}$ and the competitive antiandrogen cyproterone acetate (CPA) resulted in a complete block of imposex development. This indicates that EE_2 does not induce imposex directly but mediated by androgens. Based on these results it has been assumed that exposure of snails to EE_2 concentrations $\geq 0.25 \mu\text{g l}^{-1}$ causes an increase of endogenous androgen titers, most probably due to an inhibition of the cytochrome P450-dependent aromatase (Schulte-Oehlmann et al. 2001a). Some evidence for this was found in EE_2 exposed ramshorn snails exhibiting a mean tissue testosterone concentration of 1.75 ng g^{-1} wet weight compared to 1.31 ng g^{-1} wet weight in the control. A comparable indirect androgenic effect of oestradiol and EE_2 was reported by Quaglino et al. (2002) for female zebra finches exhibiting male song behavior as a consequence of aromatase inhibition.

Fig. 19.3. *Marisa cornuarietis*. Development of the vas deference sequence index (VDSI) as a measure of imposex intensities in snails exposed to EE₂ (a) and MT (b) during the AE experiment series. Exposure groups: (×) solvent control; (○) 0.1 μg l⁻¹; (△) 0.25 μg l⁻¹; (▽) 0.5 μg l⁻¹; (□) 1.0 μg l⁻¹ (n = 30 snails per group)



Bögi et al. (2002) demonstrated that a treatment by exogenous oestrogens elevated both, oestrogen and androgen receptor mRNA in the African claw frog *Xenopus laevis*, indicating stimulatory functions of oestrogens for gene expression of both receptors. Effects on sexual differentiation during larval development were achieved by treatment with EE₂ and the antiandrogen cyproterone acetate both causing feminization, the anti-oestrogen tamoxifen resulting in neutralization, and the androgens, MT and dihydrotestosterone, but not testosterone, leading to masculinization. A virilization of female *Carassius carassius* by exposure to aqueous MT concentrations between 0.01 and 1 μg l⁻¹ was also achieved in the experiments of Fujioka (2002) and confirmed in the study of Zerulla et al. (2002) for fathead minnows (*Pimephales promelas*) in the concentration range from 10 to 100 μg l⁻¹. The synthetic androgen caused a degeneration of the albumen gland in the pond snail, *Lymnaea stagnalis* (Czech et al. 2001), although the authors did not observe any adverse effects on fecundity or fertility at concentrations from 1 to 100 ng MT l⁻¹.

In the amphipod *Hyalella azteca* EE₂ exposure to concentrations from 0.1 to 0.32 μg l⁻¹ resulted in the development of significantly smaller second gnathopods of males (Vandenbergh et al. 2003). Furthermore, histological aberrations of the reproductive

tract, indications of hermaphroditism, disturbed maturation of germ cells, and disturbed spermatogenesis were observed in males at all EE₂ exposures. This indicates, that at least in prosobranch snails and crustaceans, EE₂ and MT have comparable effects as in vertebrates while the results for other invertebrate taxa are less unequivocal. Tests performed with the synthetic oestrogen and the midge *Chironomus riparius* did not affect sexual differentiation or fecundity parameters (Watts et al. 2003).

EE₂ and MT did not only cause a significant virilization of females in the tested concentration range but affected also the formation of germ cells in male and female gonads. Even when kept in the laboratory under constant conditions, reproduction in *M. cornuarietis* is characterized by a marked seasonality. The spawning activity of females increases in October and the main spawning period extends until March while during the rest of the year by far less clutches and eggs are produced. This indicates that a typical sexual repose phase without any reproduction is lacking in *Marisa*. We can rather distinguish phases of differing reproductive activity during the year but not a total cessation of breeding.

In the ovary, oocytes derive from oogonia which are part of the germinal epithelium. In the last phase of oogenesis, yolk is incorporated into the previtellogenic oocytes and finally, the ripe postvitellogenic oocytes detach from the germinal epithelium of the follicle (Fig. 19.4a). During this process, a small percentage of oocytes may degenerate with a consecutive resorption of yolk and cellular material by phagocytes (Linke 1933). Normally, this degeneration occurs only at the end of the spawning season and is typically initiated with a detachment of the egg membrane and a following release of plasma and yolk into the follicle's lumen.

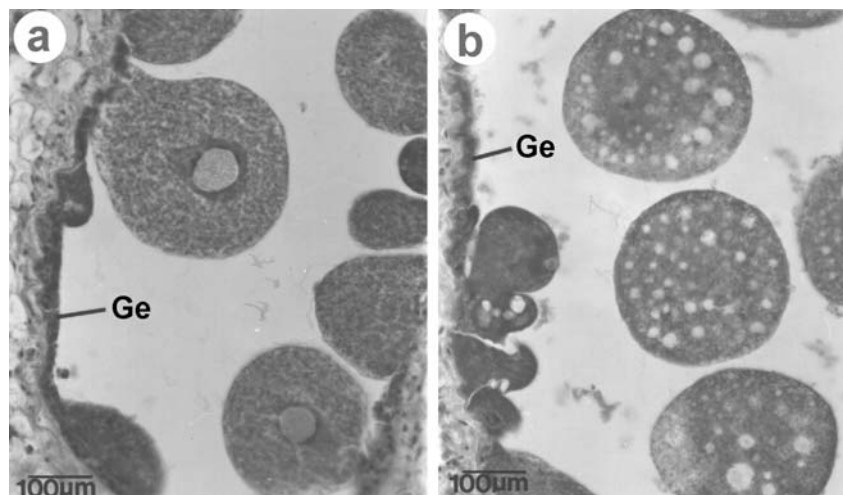


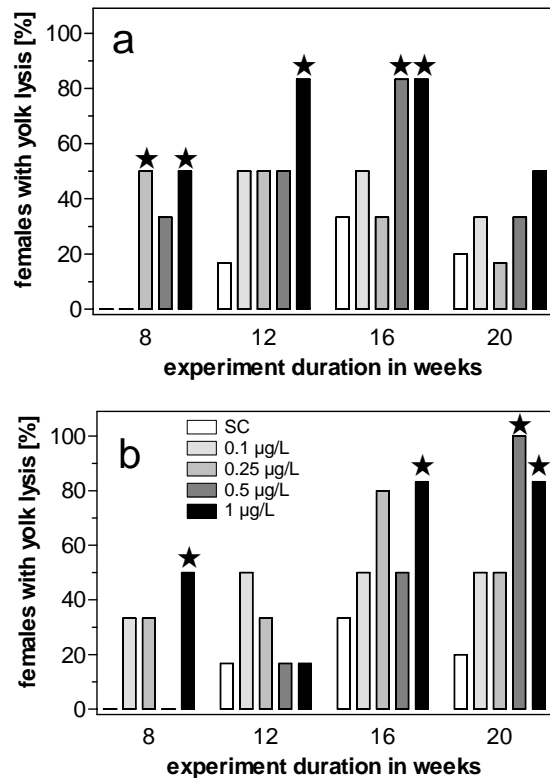
Fig. 19.4. *Marisa cornuarietis*. Histological photographs of the ovary; **a** control female: ovarian follicle with normal oogenesis and ripe postvitellogenic oocytes detaching from the germinal epithelium (*Ge*); **b** EE₂ exposed female: Extensive yolk lysis in postvitellogenic oocytes, indicated by yolk extrusion in postvitellogenic oocytes

In groups exposed to either EE₂ or MT, the incidence of females with a massive yolk lysis in postvitellogenic oocytes (Fig. 19.4b) and a consecutive resorption of female germ cells was generally higher when compared with the control group (Fig. 19.5). There was a tendency towards a more pronounced effect at higher EE₂ and MT concentrations but the two synthetic steroids impaired oogenesis for a different period of the experiment.

While in the control group yolk lysis occurred only from week 12 to 24 with a maximum incidence of 33%, the effect could be detected throughout the experiment in the EE₂ exposed groups with an incidence of up to 83% and from week 8 to 24 under MT exposure for an incidence of up to 100%. The fecundity was reduced in those groups exposed to the two highest EE₂ or MT concentrations because these females produced up to 60% less eggs compared to control females.

The interference of EE₂ and MT with oogenesis was confirmed for the JE experiment but with slightly different effects. In juvenile and sexually still immature females atretic oocytes with a complete resorption of the germ cells were observed for almost all specimens on a regular basis while a yolk lysis, as indicated by the white colored extrusions in the gem cells (Fig. 19.4b), occurred only occasionally.

Fig. 19.5. *Marisa cornuarietis*. Incidence of yolk lysis and oocyte resorption in females exposed to EE₂ (a) and MT (b) during the AE experiment series. Asterisks indicated statistically significant differences compared to the control ($p < 0.05$, χ^2 test, $n = 6$ snails per group). SC, solvent control



An impairment of oogenesis, caused by endocrine disrupting chemicals, is likewise reported for aquatic vertebrates with the example of female rainbow trouts, *Oncorhynchus mykiss*, following an exposure to the PCB mixture Arochlor 1260 (Matta et al. 1998). 18.2% of the treated females exhibited severe gonad anomalies and a reduced development of oocytes.

19.3.2

Effects on Males at Concentrations from 0.1 to 1 $\mu\text{g l}^{-1}$

Both test compounds affected also the spermatogenesis in male snails although at an earlier phase of the experiment. This could be expected because generally spermatogenesis proceeds oogenesis in species with internal fertilization. Males have to produce viable sperm in advance to copulate with the females before the onset of female spawning.

Fig. 19.6 summarizes the different stages of spermatogenesis impairment in *M. cornuarietis*. The stages represent the basis of the spermatogenesis disturbance index (SDI), calculated as the mean value of stages within an exposure group. In stage 0 spermatogenesis is unaffected and sperm can be observed in all tubuli seminiferi (Fig. 19.6a). Stage 1 is characterized by an oligospermia with a reduced density of all spermatogenesis stages (Fig. 19.6b). In stage 2 which is defined as an azoospermia, a complete breakdown of spermatogenesis can be observed with a total lack of ripe sperm (Fig. 19.6c). Finally, the testis degenerates and the germinal epithelium of the tubuli seminiferi is no longer intact (stage 3, Fig. 19.6d). It seems to be unlikely that males exhibiting stages 2 and 3 of spermatogenesis impairment are able to reproduce. Consequently, an increase of the SDI to values above 1.0 indicates a reduced male reproductive success in exposure groups.

During the AE experiment series the SDI in the control group never exceeded a value of 0.5 while in the groups exposed to EE₂ and MT the maximum values measured were 1.83 and 1.50, respectively (Fig. 19.7). Already the lowest nominal concentrations of both synthetic steroids applied in the tests resulted in a significant increase of the SDI after 1 and 8 weeks but generally, EE₂ was more effective when compared with MT regarding spermatogenesis as a toxicological endpoint.

These findings demonstrate that an exposure to potent androgen receptor agonists such as MT do not result in the development of “supermales”, an observation which is in line with reported effects of androgenic compounds in mammalian species, including humans. Men treated with anabolic steroids are characterized not only by a reduced testis size but also by a massive impairment of spermatogenesis and in extreme cases by a complete loss of reproducing capability (O'Sullivan et al. 2000). Comparable results were already reported by Wilson and Wilson (1943) in their early experiments with rats.

19.3.3

Effects of EE₂ at Concentrations from 1 to 100 ng l^{-1}

During the LC experiment series with its lower EE₂ concentration range tested no indication for an increase of the VDSI as a measure of imposex intensity was observed (Fig. 19.8). The variability within and between EE₂ exposed and control groups was

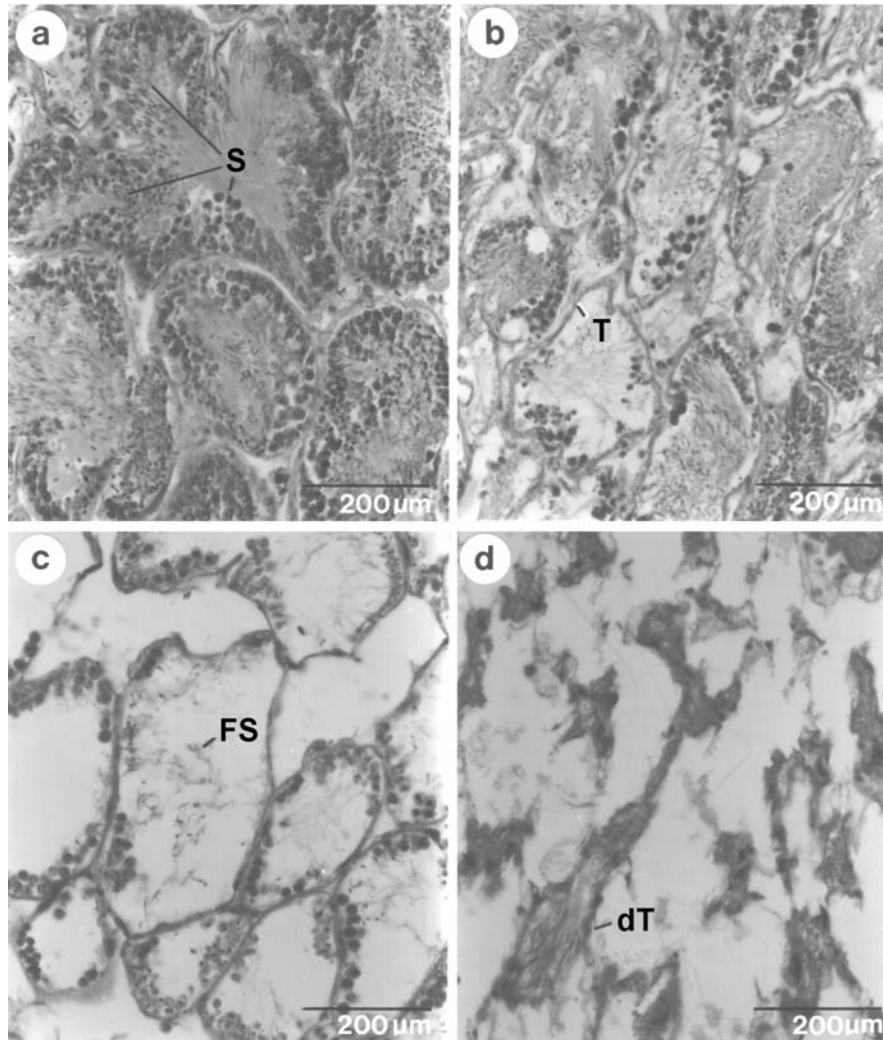


Fig. 19.6. *Marisa cornuarietis*. Histological photographs of the testis, demonstrating the different stages of the spermatogenesis disturbance index (SDI); **a** Stage 0: normal spermatogenesis; **b** Stage 1: oligospermia with significantly reduced spermatogenesis; **c** Stage 2: azoospermia with breakdown of spermatogenesis; **d** Stage 3: testis degeneration with structural collapse of the organ. *dT*, degenerating testis tubule; *FS*, fragments of disintegrated sperm; *S*, different spermatogenesis stages; *T*, testis tubule

remarkably low with no statistically significant difference ($p > 0.05$, Weir test). The results of all three EE_2 experiment series – AE, JE and LC – are in good agreement. Obviously, a minimum nominal concentration of $0.25 \mu\text{g } EE_2 \text{ l}^{-1}$ is required to induce imposex in *M. cornuarietis* with the no effect concentration being $0.1 \mu\text{g l}^{-1}$ (cf. Fig. 19.3a). Because the latter was the highest test concentration in the LC experiment, no imposex development was observed.

Fig. 19.7. *Marisa cornuarietis*. Incidence and severity of spermatogenesis impairment, measured by the spermatogenesis disturbance index (SDI), in males exposed to EE2 (a) and MT (b) during the AE experiment series. Asterisks indicated statistically significant differences compared to the control ($p < 0.05$, χ^2 test, $n = 6$ snails per group). SC, solvent control

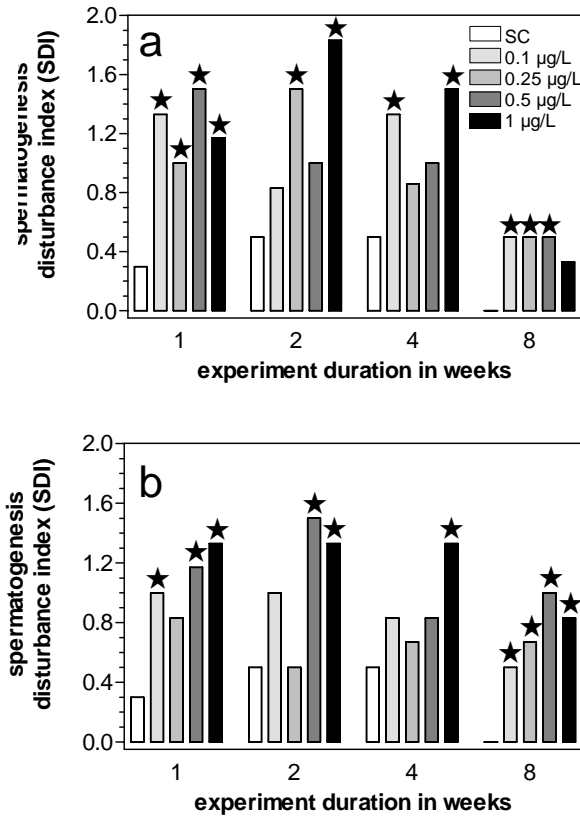
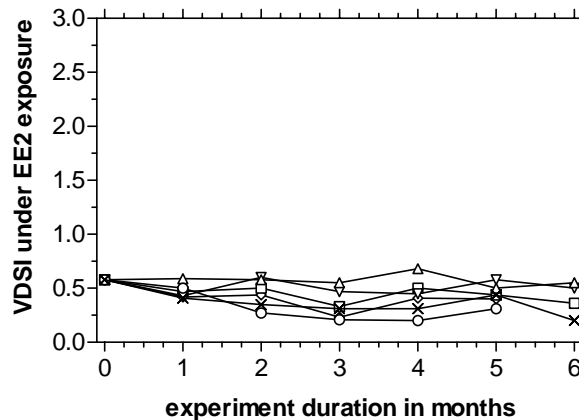


Fig. 19.8. *Marisa cornuarietis*. Development of the vas deference sequence index (VDSI) as a measure of imposex intensities in snails exposed to ethinyloestradiol during the LC experiment series. Exposure groups: (×) solvent control; (○) 1 ng l⁻¹; (△) 10 ng l⁻¹; (▽) 25 ng l⁻¹; (□) 50 ng l⁻¹; (◇) 100 ng l⁻¹ ($n = 30$ snails per group)



Although the synthetic oestrogen did not induce a virilization of females at concentrations of up to 100 ng l^{-1} , EE_2 exhibited other, not less severe effects. The oestrogen induced a complex syndrome of morphological and physiological alterations in females which is referred to as the induction of “superfemales”.

Superfemales in *M. cornuarietis* were first described by Oehlmann et al. (2000) and found in experiments with the xeno-oestrogens bisphenol A (BPA) and octylphenol (OP). Affected specimens are characterized 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. Superfemales with enlarged female sex glands or malformed oviducts were found in all groups exposed to EE_2 . Almost all females in the groups receiving the synthetic steroid were characterized by an enlarged pallial gland complex but the incidence of pathomorphological changes of albumen or capsule glands was much lower. The highest proportion was found in the groups receiving 1 or $10 \text{ ng EE}_2 \text{ l}^{-1}$ with an incidence of 2.3% and 2.4%, respectively, while the corresponding values were generally below 1% in all other experimental groups exposed to EE_2 . This indicates that the lowest concentrations were more effective in inducing superfemales than the higher ones during the LC experiment. This result offers an explanation why superfemales did not occur during the AE and JE experiments because during these two series the applied concentrations were too high to induce superfemales. Superfemales were never observed in control groups or in experiments testing non-oestrogenic compounds.

Gross malformations of the pallial oviduct, such as ruptures in the transition zone between albumen and capsule gland in *M. cornuarietis*, are believed to be a consequence of a massive stimulation of egg and clutch production in females exposed to oestrogenic compounds (Oehlmann et al. 2000; Schulte-Oehlmann et al. 2001a, b). If egg production of the different EE_2 exposed groups is compared with the solvent control during the entire LC experiment, it is obvious that only in the $10 \text{ ng EE}_2 \text{ l}^{-1}$ group a slight and statistically insignificant increase of the cumulative egg number was found ($p > 0.05$, ANCOVA). In the other EE_2 exposed groups a significantly lower number of eggs was produced when compared with the control ($p < 0.05$, ANCOVA; Fig. 19.9a). But it has to be considered that the experimental period covered the main spawning season of ramshorn snails.

A separate evaluation of the different phases of the reproductive cycle during the experiment results in inconsistent findings, as indicated in Fig. 19.9b for the time before and in Fig. 19.9c during the spawning period. Before the onset of main spawning activities in the control group, females exposed to either 10 or $25 \text{ ng EE}_2 \text{ l}^{-1}$ produce significantly more eggs than control females while snails exposed to $50 \text{ ng EE}_2 \text{ l}^{-1}$ produce significantly fewer eggs with the two remaining experimental groups exhibiting no differences to the control group (ANCOVA, Fig. 19.9b). Obviously, EE_2 is capable to induce superfemales outside the spawning season only in a rather small concentration window with concentrations of up to 25 ng l^{-1} being more effective than higher concentrations. These findings are in line with the occurrence of superfemales exhibiting gross malformations of the pallial oviduct section at this phase of the experiment.

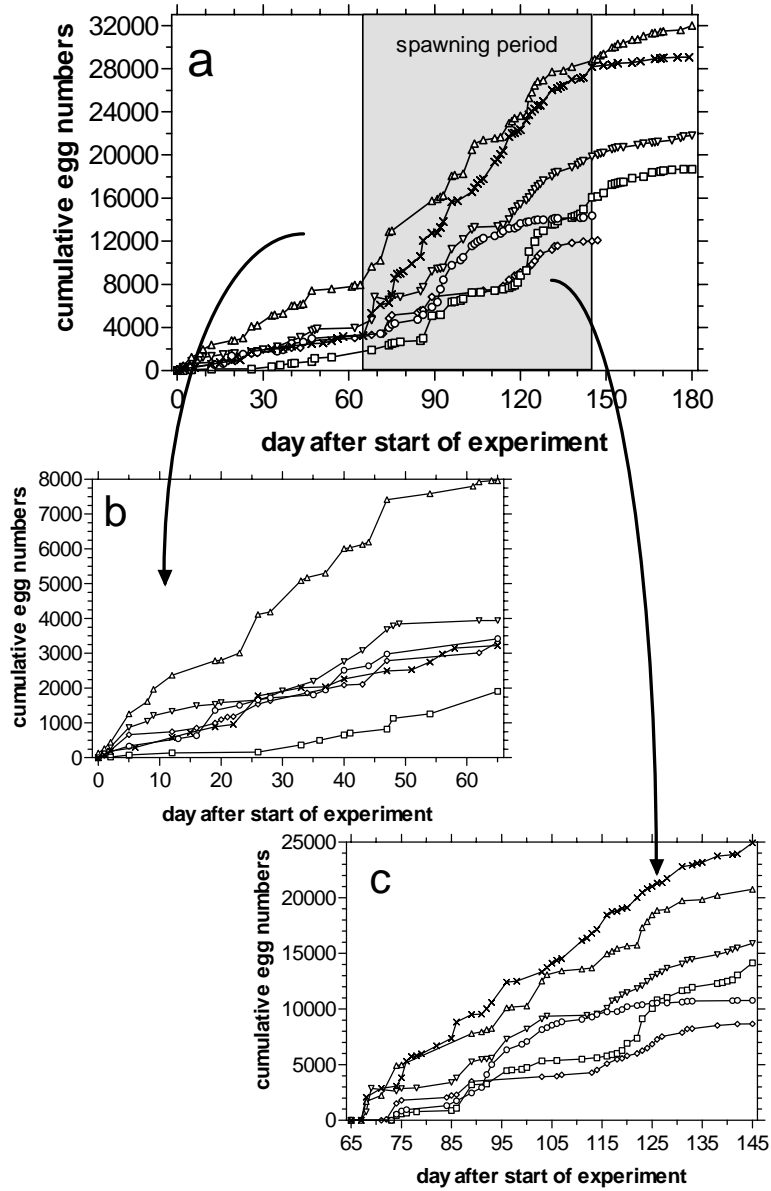


Fig. 19.9. *Marisa cornuarietis*. Cumulative numbers of eggs produced by all females in groups exposed to ethinyloestradiol during the LC test series. Egg production over the entire length of the experiment (a) and furthermore considering only the experimental period before (b) and during the spawning period (c) are shown. Exposure groups: (×) solvent control; (○) 1 ng l⁻¹; (△) 10 ng l⁻¹; (▽) 25 ng l⁻¹; (□) 50 ng l⁻¹; (◇) 100 ng l⁻¹

During the spawning season control females produced about four times more eggs compared to the pre-spawning period (Fig. 19.9c). At this phase of the experiment no superfemales with a rupture in the pallial oviduct section did occur but all EE₂ exposed groups were characterized by a statistically significant reduced fecundity compared to the control ($p < 0.05$, ANCOVA). Surprisingly, the fecundity-reducing effect of the synthetic steroid was most pronounced at the lowest and highest concentration tested and in these two groups the mortality increased significantly ($p < 0.001$, χ^2 test). None of the snails survived the termination of the spawning season in these two groups.

The results for the LC experiment series indicate that it is of crucial importance for the toxicological characterization of endocrine active compounds to consider not only the tested concentration range but furthermore the reproductive stage of the test organisms during the experiment. It has also been shown for other endocrine active substances such as BPA and OP that typical oestrogenic effects can be partially or totally masked during the spawning season of the animals (Schulte-Oehlmann et al. 2001b). Comparable results were found in other aquatic organisms, including vertebrates. Jobling et al. (1996) and Blázquez et al. (1998) reported that the effects of alkylphenols, natural and synthetic oestrogens on the gonads of male fish were dependent from the phase of the reproductive cycle and varied considerably if the exposure experiments were conducted before, during or after the spawning season.

19.3.4

Synopsis of Effects Data for EE₂ and MT in *Marisa cornuarietis*

Table 19.1 summarizes the NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) values determined for EE₂ and MT during the different test series with *M. cornuarietis*. It has to be considered, that so far no experiments were performed with ramshorn snails and MT in the concentration range below 0.1 $\mu\text{g l}^{-1}$.

Table 19.1. *Marisa cornuarietis*. Overview of NOEC and LOEC values obtained for EE₂ and MT and the different toxicological endpoints in laboratory tests considering the tested nominal concentration range from 0.001 to 1 $\mu\text{g EE}_2 \text{l}^{-1}$ and from 0.1 to 1 $\mu\text{g MT l}^{-1}$

Endpoint	Ethinylloestradiol		Methyltestosterone	
	NOEC [ng l^{-1}]	LOEC [ng l^{-1}]	NOEC [ng l^{-1}]	LOEC [ng l^{-1}]
Imposex	100	250	?	100
Superfemales	1	10	—	—
Reduced fecundity	?	1	250	500
Oogenesis	100	250	250	500
Spermatogenesis	?	100	?	100

—, Effect not observed; ?, not determined because below the tested concentration range.

The two adverse effects observed at the lowest EE₂ concentrations were the induction of superfemales with a LOEC of 10 ng l⁻¹ and the reduced fecundity of ramshorn snails during the main spawning season with a LOEC of only 1 ng l⁻¹. The latter value is identical with effect concentrations observed in toxicological experiments with fish (Young et al. 2002), ending up with a predicted no effect concentration (PNEC) as low as 0.4 ng l⁻¹ which now can be confirmed for aquatic invertebrates with the example of *M. cornuarietis*. Based on the so far conducted tests with snails and MT, it can be expected that prosobranch mollusks might already be affected at concentrations below 100 ng l⁻¹ due to the compound's ability to induce imposex and to impair spermatogenesis but this has to be investigated in detail in future experiments.

Another question of interest is whether or not EE₂ and MT pose a risk for the survival of snail populations in the field considering concentrations of both compounds which have been measured in the environment. No reports providing MT concentrations in surface waters are available while the occurrence of EE₂ in both, effluents of sewage treatment works and surface waters, has been widely investigated (for review cf. Halling-Sørensen et al. 1998; Ternes 1998; Belfroid et al. 1999; Ternes et al. 1999; Williams et al. 1999, 2003). Generally, EE₂ concentrations in effluent samples are below 80 ng l⁻¹ while maximum concentrations in surface waters were 4.3 ng l⁻¹ and 3.4 ng l⁻¹ for the Netherlands and the United Kingdom, respectively (Belfroid et al. 1999; Williams et al. 2003).

The comparison of measured EE₂ residues in surface waters and effect concentrations for this synthetic steroid in laboratory experiments using *M. cornuarietis* as a test species indicates clearly that prosobranch populations in the field can be affected by this oestrogenic pharmaceutical.

Acknowledgments

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References

- Belfroid AC, Van der Horst A, Vethaak AD, Schafer AJ, Rijs GBJ, Wegener J, Cofino WP (1999) Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci Total Environ* 225: 101-108
- Blázquez M, Zanuy S, Carrillo M, Piferrer F (1998) Structural and functional effects of early exposure to estradiol-17β and 17α-ethynylestradiol on the gonads of the gonochoristic teleost *Dicentrarchus labrax*. *Fish Physiol Biochem* 18: 37-47
- Bögi C, Levy G, Lutz I, Kloas W (2002) Functional genomics and sexual differentiation in amphibians. *Comp Biochem Phys B* 133: 559-570
- Czech P, Weber K, Dietrich DR (2001) Effects of endocrine modulating substances on reproduction in the hermaphroditic snail *Lymnaea stagnalis* L. *Aquat Toxicol* 53: 103-114
- Daughton CG, Ternes TA (1999) Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ Health Perspect* 107 (Suppl 6): 907-938
- Duft M, Schulte-Oehlmann U, Tillmann M, Markert B, Oehlmann J (2003a) Toxicity of triphenyltin and tributyltin to the freshwater mudsnail *Potamopyrgus antipodarum* in a new sediment biotest. *Environ Toxicol Chem* 22: 145-152
- Duft M, Schulte-Oehlmann U, Weltje L, Tillmann M, Oehlmann J (2003b) Stimulated embryo production as a parameter of estrogenic exposure via sediments in the freshwater mudsnail *Potamopyrgus antipodarum*. *Aquat Toxicol* 64:437-449

- Fujioka Y (2002) Effects of hormone treatments and temperature on sex-reversal of Nigorobuna *Carassius carassius grandoculis*. *Fish Sci* 68: 889-893
- Halling-Sørensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE (1998) Occurrence, fate and effects of pharmaceutical substances in the environment - a review. *Chemosphere* 36: 357-393
- Jobling S, Sheahan D, Osborne JA, Matthiessen P, Sumpter JP (1996) Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ Toxicol Chem* 15: 194-202
- Kümmerer K (ed) (2001) Pharmaceuticals in the environment. Sources, fate, effects and risks. Springer, Berlin, Heidelberg, New York
- Linke O (1933) Morphologie und Physiologie des Genitalapparates der Nordseelittorinen. *Wiss Meeresunters Abt Helgoland* 19: 1-60
- Lozán JL (1992) *Angewandte Statistik für Naturwissenschaftler*. Parey, Berlin Hamburg
- Matta MB, Cairncross C, Kocan RM (1998) Possible effects of polychlorinated biphenyls on sex determination in rainbow trout. *Environ Toxicol Chem* 17: 26-29
- Mensink BP, Everaarts JM, Kralt H, ten Hallers-Tjabbes CC, Boon JP (1996) Tributyltin exposure in early life stages induces the development of male sexual characteristics in the common whelk, *Buccinum undatum*. *Mar Environ Res* 42: 151-154
- Oehlmann J, Schulte-Oehlmann U (2003) Endocrine disruption in invertebrates. *Pure Appl Chem* 75:2207-2218
- Oehlmann J, Schulte-Oehlmann U, Tillmann M, Markert B (2000) Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part I: Bisphenol A and octylphenol as xeno-estrogens. *Ecotoxicology* 9: 383-397
- O'Sullivan AJ, Kennedy MC, Casey JH, Day RO, Corrigan B, Wodak AD (2000) Anabolic-androgenic steroids: Medical assessment of present, past and potential users. *Med J Australia* 173: 323-327
- Quaglini AE, Craig-Veit CB, Viant MR, Erichsen AL, Fry DM, Millam JR (2002) Oral estrogen masculinizes female zebra finch song system. *Horm Behav* 41: 236-241
- Schulte-Oehlmann U, Bettin C, Fioroni P, Oehlmann J, Stroben E (1995) *Marisa cornuarietis* (Gastropoda, Prosobranchia): a potential TBT bioindicator for freshwater environments. *Ecotoxicology* 4: 372-384
- Schulte-Oehlmann U, Watermann B, Tillmann M, Scherf S, Markert B, Oehlmann J (2000) Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part II: Triphenyltin as a xeno-androgen. *Ecotoxicology* 9: 399-412
- Schulte-Oehlmann U, Markert B, Oehlmann J (2001a) Development of a biotest with *Marisa cornuarietis* (Gastropoda: Prosobranchia) for the assessment of environmental chemicals with sex hormone-mimicking effects. Final report for R&D project 297 65 001/04, Federal Environmental Agency, Berlin
- Schulte-Oehlmann U, Tillmann M, Casey D, Duft M, Markert B, Oehlmann J (2001b) Östrogenartige Wirkungen von Bisphenol A auf Vorderkiemerschnecken (Mollusca: Gastropoda: Prosobranchia). *UWSF - Z Umweltchem Ökotox* 13: 319-333
- Ternes TA (1998) Occurrence of drugs in German sewage treatment plants and rivers. *Wat Res* 32: 3245-3260
- Ternes TA, Hirsch R, Stumpf M, Eggert T, Schuppert B, Haberer K (1999) Nachweis und Screening von Arzneimittelrückständen, Diagnostika und Antiseptika in der aquatischen Umwelt. Final report for BMBF R&D project 02WU9567/3. ESWE, Wiesbaden
- Vandenbergh GF, Adriaens D, Verslycke T, Janssen CR (2003) Effects of 17 α -ethinylestradiol on sexual development of the amphipod *Hyaella azteca*. *Ecotox Environ Saf* 54: 216-222
- Watts MM, Pascoe D, Carroll K (2003) Exposure to 17 α -ethinylestradiol and bisphenol A-effects on larval moulting and mouthpart structure of *Chironomus riparius*. *Ecotox Environ Saf* 54: 207-215
- Williams RJ, Jürgens MD, Johnson AC (1999) Initial predictions of the concentrations and distribution of 17 β -oestradiol, oestrone and ethinyl oestradiol in 3 English rivers. *Wat Res* 33: 1663-1667
- Williams RJ, Johnson AC, Smith JLL, Kanda R (2003) Steroid estrogens profiles along river stretches arising from sewage treatment works discharges. *Environ Sci Technol* 37: 1744-1750
- Wilson JG, Wilson HC (1943) Reproductive capacity in adult rats treated prepubertally with androgenic hormone. *Endocrinology* 33: 350-353
- Young WF, Whitehouse P, Johnson I, Sorokin N (2002) Proposed predicted-no-effect-concentrations (PNECs) for natural and synthetic steroid oestrogens in surface waters. Research and Development, Technical Report P2-T04/1, Environment Agency, Bristol
- Zerulla M, Länge R, Steger-Hartmann T, Panter G, Hutchinson T, Dietrich DR (2002) Morphological sex reversal upon short-term exposure to endocrine modulators in juvenile fathead minnow (*Pimephales promelas*). *Toxicol Lett* 131: 51-63