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Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent☆

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Abstract

It is now well established that there is a diverse array of chemicals discharged into the environment that can mimic or antagonise the action of hormones. These endocrine-disrupting chemicals (EDCs) can thus interact with physiological systems and cause alterations in development, growth and reproduction in wildlife that are exposed to them. As yet, however, there is little information on the relative sensitivities of different wildlife groups to these chemicals and/or mixtures of them (e.g. estrogenic effluents) and hence, there are fundamental shortfalls in our knowledge of the ecological importance of endocrine disruption in wildlife. In this study, the effects of exposure to individual estrogenic chemicals (17α-ethinylestradiol; EE_2, bisphenol-A, and 4-*tert* octylphenol) and a mixture containing these chemicals (treated sewage effluent) on embryo production in the prosobranch mollusc, *Potamopyrgus antipodarum*, were studied and compared with the effects of EE_2 and the same estrogenic effluent on vitellogenin induction and/or egg production in various species of freshwater fish (fathead minnow; *Pimaphales promelas*, rainbow trout (*Oncorhynchus mykiss*); *Cyprinus carpio*, carp; *Cyprinus carpio*). The lab-based studies demonstrated that all of the tested chemicals (known to be estrogenic and to cause reproductive effects in fish) also affected embryo production in *P. antipodarum*. Furthermore, exposure to EE_2 induced similar reproductive responses in the snails as in the fathead minnow (*Pimephales promelas)*, stimulating egg/embryo production at low doses (up to 1 ng/l in the minnow and 25 ng/l in the snail) and causing inhibitory effects at higher doses. A similar pattern of embryo production occurred in *P. antipodarum* when it was exposed to a graded concentration of treated sewage effluent containing mixtures of estrogenic EDCs and hence, the total number of new embryos produced by the snails increased steadily over the 9 weeks exposure period in treated snails. Plasma vitellogenin concentrations in two species of male fish (the rainbow trout and the carp) also increased over the same time period. These data indicate that both the nature of the response and the relative sensitivities to environmental estrogens in *P. antipodarum* and three different

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fish species fish are comparable. *P. antipodarum* is thus, potentially a sensitive test organism for assessing estrogenicity of chemicals with a relevance to their activity in vertebrates.

Keywords: Snails; Prosobranch; Estrogen; Fish; Ethinylestradiol

1. Introduction

Environmental estrogens are chemical pollutants that can disrupt the endocrine system of animals by binding to and activating the estrogen receptor(s). They include both natural and synthetic steroid estrogens, as well as a variety of estrogen-mimicking chemicals, such as 4-nonylphenol, bisphenol-A (BPA) various plasticizers, herbicides and pesticides (Colborn et al., 1993). It is now generally accepted that endocrine-disrupting chemicals (EDCs), including environmental estrogens, are at least partially responsible for disruption of reproduction and development in some wildlife populations (Tyler et al., 1998; Vos et al., 2000). The effects found include altered/abnormal blood hormone levels, reduced fertility and fecundity, masculinization of females and feminisation of males. Global efforts are now underway to develop a greater understanding of how EDCs disrupt physiological function and to develop regulatory tests for EDCs that are broadly applicable to a variety of wildlife species (CSTEE, 1999; Campbell et al., 1999; Kavlock et al., 1996). Information on the impacts of EDCs on wildlife populations is, however, limited to a few species in several vertebrate and invertebrate taxa. Furthermore, the responses and relative sensitivities of different animal species to EDCs have not been comprehensively compared and may vary, both within and between taxa. Consequently, there are fundamental shortfalls in our knowledge of the relative importance of endocrine disruption in different wildlife taxa, and of the level of the problem of endocrine disruption in wildlife relative to other environmental stressors.

Most of the evidence for endocrine disruption in wildlife has come from studies on species living in, or closely associated with, the aquatic environment, which is perhaps not surprising given the fact that our rivers and oceans act as repositories for the discharge of tens of thousands of chemicals in large volumes. The majority of laboratory-based studies on EDCs in vertebrates have focused on the effects of estrogenic chemicals, because many of the effects seen in vertebrate wildlife are believed to have resulted from disruption of this axis. In vertebrates, estrogens play a fundamental role in both reproduction and somatic cell function, sexual differentiation, the development of secondary sex characteristics, ovulation, the regulation of mating and breeding behaviours, and the regulation of calcium and water homeostasis (Fairbrother, 2000). In adult fish, for example, exposure to xenoestrogens, or to synthetic or natural estrogens, has been reported to result in altered fecundity in female fish (Giesy et al., 2000; Lange et al., 2001; Sohoni et al., 2001), reduced testicular development (e.g. Jobling et al., 1996; Gimeno et al., 1998) and fertility (Jobling et al., 2002) in male fish, and in increased or decreased vitellogenin production (the precursor of egg yolk protein) in both male and female fish (e.g. Kramer et al., 1998; Miles-Richardson et al., 1999; Scholz and Gutzeit, 2000). To date, very few studies have compared the effects of estrogens and estrogenic effluents in different vertebrates in the same taxon (fish or otherwise), or between different taxa (Thompson et al., 2000).

Invertebrate models for assessing endocrine effects are very much needed, both for developing knowledge on the potential impact of EDCs on invertebrate populations, and to determine commonalities and differences between vertebrate and invertebrate responses to EDCs. Few studies have, however, examined the effects of EDCs on invertebrates, mainly due to the general lack of knowledge of their basic endocrine physiology. Moreover, in the better studied invertebrate taxa (the crustacea and the insecta), it seems that many of the physiological functions that are under hormonal control, have no vertebrate comparison (DeFur et al., 1999). Although vertebrate-like sex steroid hormones have been

discovered in many invertebrate groups, their function, in most cases remains equivocal. Only in molluscs (slugs and snails) has a role for vertebrate type sex steroids been suggested (Bettin et al., 1996; Geraerts and Joosse, 1984). If this is the case, then this invertebrate group may be sensitive to vertebrate sex steroids and their mimics. Some of the strongest data from field studies to demonstrate causes and effects of EDs are derived from prosobranch molluscs in which organotin compounds were reported to cause imposex and intersex, resulting in a virilization of female snails (in over 150 species of mollusc; Matthiessen and Gibbs, 1998; Barroso et al. 2002; Evans et al., 2001). Mechanistic studies on the induction of imposex and intersex in prosobranch molluscs indicate that steroids (particularly testosterone) may play an important role in the manifestation of these abnormalities (Bettin et al., 1996; Gooding and LeBlanc, 2001). Furthermore, imposex and intersex can be induced by exposure of prosobranch molluscs to androgens or androgen mimics. Moreover exposure of two prosobranch snails (*Marisa cornuarietis; a tropical freshwater snail, and Nucella lapillus; a marine species)* to the environmental estrogens BPA and 4-*tert* octylphenol has been shown to affect egg production, suggesting that these invertebrates are sensitive to the effects of estrogens (Oehlmann et al., 2000). The aim of this study was to compare the effects of estrogenic chemicals on reproductive responses in prosobranch molluscs and freshwater fish. This was done by assessing the effects of EE_2, 4 *tert*-octylphenol (OP), BPA) and a mixture of EDCs (in treated sewage effluent) on egg and embryo production in the prosobranch mollusc, *Potamopyrgus antipodarum* (a temperate species of mollusc abundant in freshwater ecosystemsin Europe), and comparing these responses with effects of the same effluent on vitellogenin induction in rainbow trout (*Oncorhynchus mykiss*), and carp (*Cyprinus* carpio) and with the effects of EE_2 on vitellogenin induction and egg production in fathead minnow (*Pimephales promelas*). We were not able to compare the effects of the estrogens on vitellogenin (vitellin in invertebrates) induction in the mollusc and fish, because, in *Potamopyrgus* (and other prosobranch molluscs), vitellin does not enter the haemolymph, but rather, is produced locally for uptake into the gonad.

2. Materials and methods

2.1. Animals

2.1.1. Snails

P. antipodarum stock animals were cultured in the laboratory at Zittau University, in Germany, by Professor Oehlmann and Dr Schulte-Oehlmann. These snails were used in both experiments 1 (the UK effluent study) and 3 (the laboratory exposures).

2.1.2. Fish

Immature carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) were obtained, by Professor Tyler and Dr Jobling, from commercial fish farms in Nottingham, UK (Calverton Environment Agency coarse fish farm) and Dorset, UK (Houghton Springs Fish Farm), respectively. Breeding stocks of fathead minnow between 6 and 11 months of age were kindly provided to Dr Pawloski (at the University of Heidelberg in Germany) by Dr Reinhard Länge (Schering AG, Berlin, FRG).

2.2. Chemicals for laboratory studies

17α-Ethinylestradiol (EE_2 >98% purity) and DMSO were purchased for the laboratory studies (experiments 3 and 4) from Sigma (Deisenhofen, Germany) and BPA and octylphenol from Merck Eurolab (Dresden, Germany). The snail exposures were carried out in Zittau, Germany, whilst the fathead minnow experiment was carried out in Heidelberg, Germany.

2.3. The study effluent

The effluent exposure studies were carried out in the UK. The treated sewage effluent was derived from Chelmsford Sewage Treatment Works, Chelmsford, Essex, UK. We had previously established that this effluent was estrogenic to fish (inducing vitellogenin synthesis) and that it contained a variety of environmental estrogens, including alkylphenols, BPA (Kelly, personal communication) and natural and synthetic steroid estrogens (Harries et al., 1999; Rodgers-Gray et al., 2000, 2001). The effluent contained natural steroid estrogens at concentrations ranging between 4 and 56 ng/l, and alkylphenolic chemicals, including 4-octylphenol (OP), 4-nonylphenol (NP), and

the nonylphenol mono- and diethoxylates (NP1EO and NP2EO) at concentrations between ten and 1000-fold higher than the natural steroid estrogens (in the microgram per litre range). Measured concentrations of the synthetic estrogen, ethinylestradiol (EE_2), were variable and ranged from non-detectable (<0.5 ng/l) up to 2 ng/l (Rodgers-Gray et al., 2000, 2001).

2.4. Biological endpoints measured

2.4.1. Snails

The main biological endpoints studied in *P. antipodarum* (in both laboratory exposures and in the effluent exposure study) were growth and embryo production. On each sampling occasion, between 15 and 20 snails from each treatment were anaesthetised by immersion in 2% magnesium chloride for approximately 2 h and the length of the shell (from the tip to the base) and the width of the operculum (shell opening) were measured. The shell was then cracked and the embryos within the brood pouch dissected out and counted under a stereomicroscope. The presence/absence of a shell on the embryos was recorded; unshelled embryos represent newly recruited embryos into the brood pouch (i.e. new embryo production), whilst shelled embryos are at a much later stage of development. The increase in the number of unshelled embryos, therefore, provided a measure of new embryo production.

2.4.2. Fish

2.4.2.1. Blood. Rainbow trout and carp (experiment 1) were anaesthetised with 2-phenoxyethanol (Sigma) and blood was collected via the caudal sinus using heparinised syringes containing aprotinin (2 TIU/ml). Fathead minnow (experiment 3) were transferred into a glass tank containing heparin (2000 U/l) for at least 30 min after which the fish were anaesthetised with benzocaine (Sigma) and blood was collected via caudal vein puncture, using an insulin syringe containing aprotinin (2 TIU/ml). All blood samples were centrifuged at $3000\times g$ for 10 min and the resulting plasma was stored at −80 °C prior to measurement of vitellogenin by ELISA.

2.4.2.2. Growth. The total length and weight of the fish were measured before and after the exposures. The condition factor (*K*; a measure of the body form) was calculated for individual fish as the ratio of bodyweight $(g)\times 100/(\text{total length mm})^3$.

2.4.2.3. Measurement of plasma vitellogenin. The vitellogenin content of fish plasma was quantified using either a carp (*Cyprinus carpio*) vitellogenin ELISA that has been validated for use to quantify vitellogenin in the fathead minnow and the carp (Tyler et al., 1996), or using a rainbow trout vitellogenin ELISA (Tyler et al., 2002).

2.5. Experiment one: comparative responses of fish and molluscs to a graded concentration of estrogenic sewage effluent

The objective of this experiment was to determine the responses and/or relative sensitivities of two fish species and one snail species to an estrogenic STW effluent. This was done by measuring embryo production in the snail (*P. antipodarum*) and vitellogenin production in fish (*Cyprinus carpio and Oncorhynchus mykiss*). This study was conducted with a single sewage effluent discharge (Chelmsford Essex, UK), in a continuous flow through system (in situ), in which the relative proportions of effluent and river water could be continually monitored and controlled.

Four 1 m^3 tanks were supplied with 100, 50, 25, and 12.5% sewage effluent (at ambient temperature), produced by mixing different percentages of effluent with river water. An additional two tanks were supplied with either river water alone (0% effluent; river water control) or dechlorinated tap water (absolute control). At the beginning of the experiment, each tank contained groups of 70 *P. antipodarum* (suspended from the top of each tank in small mesh enclosures), 20 sexually immature male carp and 20 sexually immature male rainbow trout. The fish and snails were exposed to the graded concentration of sewage effluent for 28 and 42 days, respectively, and sampled at time zero(0), and after 3 (fish), 7, 14, 28 (fish and snails) and 42 (snails only) days. The additional exposure time for the snails (42 days) was based on the results of laboratory studies (see experiment 2), that indicated a longer exposure period might be necessary in order to obtain a clearly defined response

on embryo production. All of the fish (up to 30 of each species) and 15 snails were sampled at each time point. Fish were fed non-estrogenic commercial trout pellets (coarse grade, Calverton fish farm UK) at 4% of their body weight, once daily, throughout the trial and *Potamopyrgus* were fed on lettuce leaves, which were renewed weekly. The tanks were aerated to ensure sufficient oxygen supply to support the biomass of fish, and the temperature was monitored daily in all tanks. Growth (fish: total length and weight. snails: shell height and operculum width), embryo production (snails) and vitellogenin production (fish) were measured and recorded at each of the specified sampling times.

2.6. Experiment two: effects of estrogens and xenestrogens on embryo production in snails

Adult snails (*P. antipodarum*) were exposed to one of three environmental estrogens, the synthetic estrogen, ethinylestradiol (EE_2), BPA, or OP for a period of 9 weeks in a semi-static renewal system in which 50% of the dosed water was replaced every 4 days. Ethinylestradiol (EE_2) and the xenoestrogens BPA and 4-*tert*-octylphenol were tested at concentrations known to be present in the aquatic environment. EE_2 concentrations in effluents in Europe range from less than detectable (<0.5 ng/l) to 62 ng/l (e.g. Belfroid et al., 1999; Desbrow et al., 1998; Ternes et al., 1999a,b.) and in surface waters at concentrations up to 5 ng/l (e.g. Huang and Sedlak, 2001). In this study, snails were dosed with EE_2 at 1, 5, 25 and 100 ng/l. Concentrations of BPA have been measured in effluents in Europe and the United States up to 25 μg/l (Belfroid et al., 2002; Staples et al., 1998), but in the ambient riverine environments, it is generally present at less than 1 μg/l. In this study, snails were dosed with BPA at 1, 5, 25 and 100 μg/l. In effluents OP is present at concentrations in the low microgram per litre range (e.g. Rippen, 1999; Blackburn and Waldock, 1996), but in surface waters, it is generally found at concentrations less than 0.2 μg/l (Guang-Guo Ying et al., 2002). Here, we dosed snails with OP at concentrations of 1, 5, 25 and 100 μg/l. Groups of 20 individual snails were examined at intervals of 0, 3, 6 and 9 weeks exposure to determine effects on growth and embryo number, as described above.

2.7. Experiment three: effects of ethinylestradiol on vitellogenin synthesis and egg production in the fathead minnow, (Pimephales promelas)

Effects of EE_2 on vitellogenin synthesis and egg production in the fathead minnow were determined in the laboratory in Heidelberg using a gonadal recrudescence assay (as described in Pawlowski et al., in press). Briefly, groups of 25 male and female fathead minnows kept previously under winter conditions (8:16 h day:night regime, 15±1 °C water temperature) for several weeks were transferred to summer conditions (16:8 h day:night regime, 25±1 °C water temperature) and immediately exposed to the EE_2 at concentrations of 0, 0.1, 1, 3,10 and 100 ng/l under continuous flow through conditions for a period of 3 weeks. Following exposures, all of the fish were blood sampled and their lengths and weights recorded and plasma samples were collected and assayed for vitellogenin, as described above.

Three pairs of fathead minnow from each of the six treatments were then transferred to 64-l aquaria with clean water, adjusted to a temperature of 25 °C and a 16:8 h light:dark regime and allowed to breed. Egg production (batch size) was recorded daily over a 3 weeks period, (eggs were removed and counted after each spawning, as described in Pawlowski et al., in press).

Fish were fed three times daily ad libitum throughout both the exposure and breeding periods with deep frozen artemia (*Artemia* sp., Fumigro, Darmstadt, FRG) and once daily with commercially available dry fish food (Tetramin Melle, FRG). Surplus food and faeces were removed twice per day.

2.7.1. Statistical analysis

Statistical significance was accepted at $P=0.05$ for all comparisons. Data sets found to lack variance homogeneity or not to have a normal distribution were log transformed prior to parametric analysis (if possible), or alternatively, analysed using non-parametric tests. Between treatment comparisons were carried out for all measured parameters by a one-way ANOVA or students *t*-test (parametric) or by a Kruskal–Wallis test or Mann–Whitney rank-sum-test (non-parametric). Subsequent multiple comparisons tests (where appropriate) were carried out using the appropriate post-hoc test. Linear regression analyses were conducted to

investigate dose–response relationships between estrogen/effluent concentration and shell height (snails), condition factor (fish), plasma vitellogenin concentration (fish), or number of eggs/embryos (fish and snails).

3. Results

3.1. Experiment one: comparative responses of fish and snails to a graded concentration of estrogenic sewage effluent

3.1.1. Measured concentrations of treated sewage effluent

The actual concentrations of the treated sewage effluent to which the animals were exposed were 100±0, 48.9±1.3, 24.2±1.2, 12.1±0.8 and 0±0% (mean percentage concentration±standard error of the mean).

3.1.2. Effects on growth and mortality

There was no snail mortality over the course of the exposure. Mortalities did occur in the two fish species, although in neither case was this related to the dose of the effluent. There was, however, an increased rate of mortality throughout the duration of the experiment (even in the control treatment; see Table 1). The reason for this is unknown. The surviving fish, however, grew consistently throughout the trial and there were no differences in the mean size (length or weight) of the fish between the treatment groups at the end of the trial ($P>0.05$; data not shown). Furthermore, the condition factor of the surviving fish did not vary across the treatments at any time point ($P>0.05$; data not shown), indicating that the surviving fish were in good health. Similarly, the snails grew well during the trial and there were no differences in the size (shell length and operculum width) between the treatment groups at the end of the trial (day 42), with the exception of the 100% effluent treatment tank, where the snails were significantly smaller than those in the other treatments at the end of the trial ($P<0.05$).

Table 1
Cumulative mortalities in groups of 20 carp (*Cyprinus carpio*) and trout (*Oncorhynchus mykiss*) exposed to 0, 12.5. 25, 50 and 100% treated sewage effluent

	Time			
Treatment	3 days	7 days	14 days	28 days
Carp				
100%	0	1	2	5
50%	0	0	0	1
25%	0	0	10	11
12.5%	0	0	1	3
0%		2	6	6
Control	0	5	5	5
Trout				
100%	0	3	4	9
50%	0	0	9	10
25%	0	3	5	5
12.5%	0	0	10	11
0%	0	0	3	4
Control	0	5	6	6

The numbers represent actual numbers of fish.

3.1.3. Plasma vitellogenin (fish)

At the start of the experiment plasma VTG concentrations in the male trout were 38±8.9 ng VTG/ml and 43±7 ng/ml in the male carp (Fig. 1a and b). After only 3 days exposure to full strength effluent, the concentrations of VTG had increased by more than tenfold in the carp (505±260 ng/ml) and by more than 200-fold in the trout (8.55±3.66 μg/ml) compared with the controls (51±7.5 ng/ml in the carp and 41±8.1 ng/ml in the trout), confirming that the effluent was estrogenic. After 14 days exposure to full strength effluent, there was a maximal induction of vitellogenin in both fish species for this effluent (130.1±19.45 μg/ml in the trout and 9.63±2.50 μg/ml in the carp), as no further increase in vitellogenin concentration was observed at day 28 in either the trout (137.8±80.97 μg/ml) or the carp (9.57±1.59 μg/ml). In the remaining effluent treatments (50, 25 and 12.5%) for the trout there was a time and dose-related induction of VTG, and the critical thresholds for a significant vitellogenic response were as follows: 7 days—50% effluent, 14 days—25% effluent, 28 days—12.5% effluent.

There was a similar pattern of response to the diluted effluents for the carp, but the threshold concentration required to induce a vitellogenic response after the longest exposure (28 days) was 25% effluent rather than 12.5% effluent (as occurred in the trout). The magnitude of the maximal vitellogenic response in the trout was also approximately tenfold higher than in the carp.

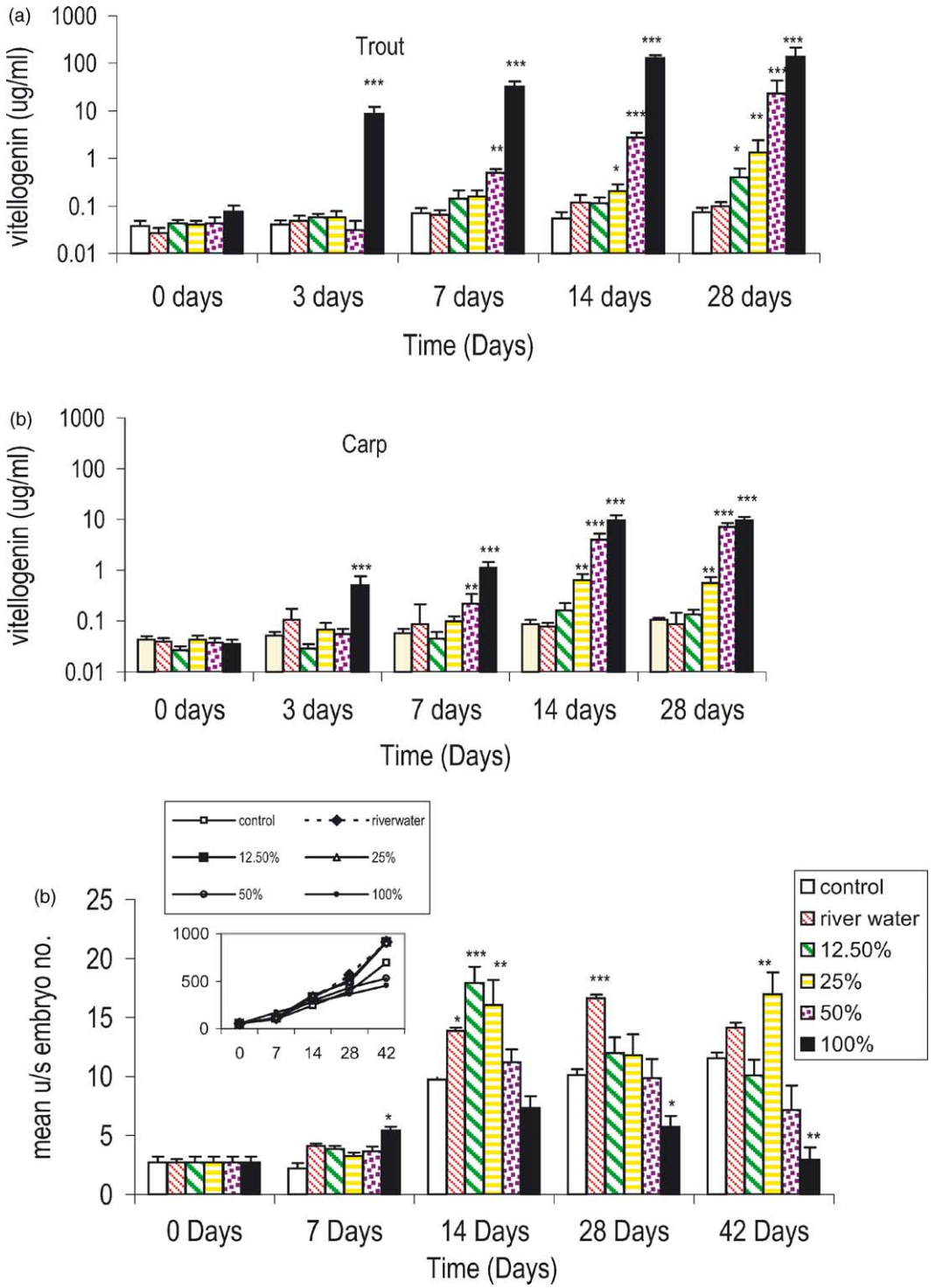

Fig. 1. Reproductive responses of snails and fish exposed to 0, 12.5, 25, 50 and 100% treated sewage effluent. (a) and (b) Cumulative vitellogenin concentrations in groups of 30 male trout (a) or carp (b) exposed to treated sewage effluents over a period of 4 weeks. (c) Both absolute and cumulative (inset) numbers of new embryos produced by groups of 20 snails (*P. antipodarum*) over a period of 6 weeks. All graphs show mean and standard error values. Asterisks indicate statistical significant differences from the control $*P<0.05$, $**P<0.01$, $***P<0.001$.

After both 3 and 7 days exposure, the mean plasma VTG concentration in carp maintained in the river water treatment appeared to be higher than in carp in the tap water (the variability of the response was also high), indicating that the river water might be estrogenic, but this was not statistically significant. No indication of estrogenic activity in the river water was, however, seen for the trout, where VTG concentrations did not differ between the river water group and the tap water controls throughout the experiment.

3.1.4. Embryo production (snails)

In *Potamopyrgus*, exposure to treated sewage effluent resulted in effects on embryo production at all concentrations tested (Fig. 1c). As with the vitellogenic response), the effect on embryo production in the snails appeared to be both time- and dose-related. In the controls (and in all other treatments), embryo production was a little lower than expected at the start of the experiment (Schulte-Oehlmann, 1997; Strzelec, 1999), although it increased fivefold relative to the start of the experiment at day 14-(in line with the seasonal reproductive cycle) and then remained at this level throughout the remaining 28 days of the experiment. At the 7 day sampling point, all effluent treatments and the river water treatment induced a stimulatory effect on embryo production, relative to the tap water control (see Fig. 1c) and the 100% effluent treatment caused the greatest stimulation of embryo production. After 14 days exposure, this effect became more pronounced in the river water, 12.5 and 25% treatments. At effluent concentrations higher than this, (e.g. 50 and 100%), however, embryo production did not differ significantly from the control rate. In the 100% effluent treatment, at this time, there was evidence for an inhibitory effect on embryo production. This effect became more pronounced with time and was significantly reduced relative to the tap water control, after 28 days exposure to 100% effluent and 42 days exposure to 100 and 50% effluent. This initial increase in embryo production (to 14 days) followed by a subsequent decrease (to 42 days) was observed for all effluent treatments. In contrast, embryo production remained constant in the controls after 14 days and for the rest of the exposure period.

3.2. Experiment two: effects of 17α-ethinylestradiol and xenoestrogens on embryo production in snails

3.2.1. Effects on growth and mortality

There were no effects of any of the chemicals (at the concentrations tested) on survival. In addition, there was little effect of any of the chemical treatments on either shell height or operculum width at any of the sampling points. The exception to this was at the final sampling point, when exposure to 5 μgBPA/l and 25 μgOP/l appeared to enhance growth-as evidenced by a significant increase in the mean shell height between 6 and 9 weeks exposure; (data not shown; $P<0.05$). At higher doses of either compound, however, this apparent stimulatory effect was not evident. EE_2 had no significant effect on the growth of the snails throughout the 9 weeks exposure.

3.2.2. Embryo production

After 3 weeks exposure, all estrogen/xenoestrogen treatments (except for 100 ng/l EE_2) caused a stimulatory effect on embryo production, relative to the control (see Fig. 2a–c) and after 42 days exposure, this effect was further pronounced. Ethinylestradiol was the most potent chemical tested; after only 21 days exposure to 1 ngEE_2/l, embryo production was significantly higher ($P=0.0138$) than in the controls. Indeed, by the end of the exposure period, the cumulative number of embryos produced in this treatment had increased relative to the control by between two and threefold. Interestingly, the shape of the dose–response curve followed an inverted U-shape, with lower concentrations (e.g. between 1 and 25 ngEE_2/l) causing an increase in embryo production relative to the controls, contrasting with an inhibitory effect on embryo production at high concentrations (e.g. 100 ngEE_2/l). The first sign of an inhibitory effect of high concentrations of EE_2 on embryo production was seen after only 3 weeks exposure.

In the snails exposed to BPA or OP, the patterns of response were similar to that observed for EE_2, albeit the concentrations needed to produce an effect were 1000-fold higher (in the microgram per litre range, rather than the nanogram per litre). After 3 weeks, snails exposed to 5 μgBPA/l, 5 μgOP/l or 25 μgBPA/l were producing significantly more embryos than the controls ($P<0.05$, 0.05 and 0.01, respectively). As

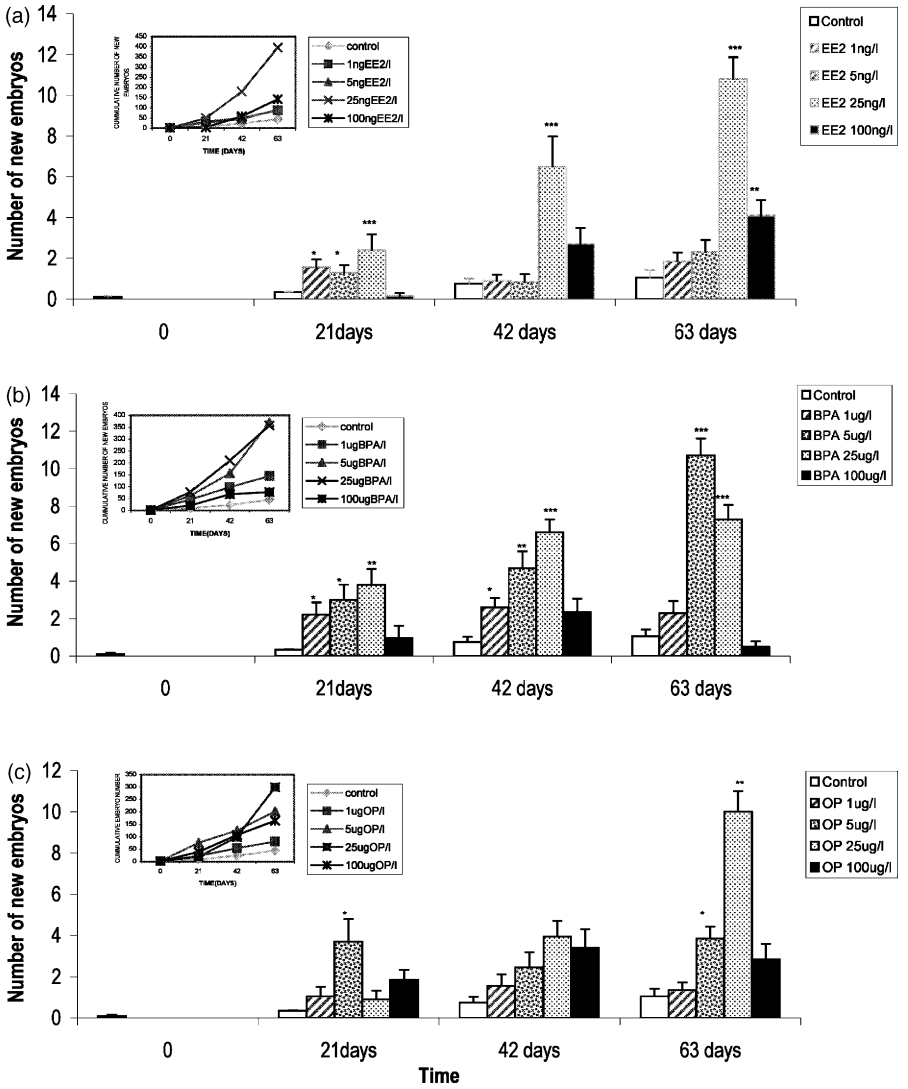

Fig. 2. *P. antipodarum* Absolute and cumulative (inset) numbers of new embryos produced by groups of snails exposed to (a) 0, 1, 5, 25 or 100 ng ethinylestradiol/l, (b) 0.1, 5, 25 or 100 μgBPA/l or (c) 0.1, 5, 25 or 100 μgOP/l. Means and standard errors are shown. Asterisks indicate statistical significant differences from the control $*P<0.05$, $**P<0.01$, $***P<0.001$.

for EE_2, higher concentrations of both BPA and OP (100 μg/l) caused an inhibition of embryo production. At the subsequent sampling points (6 and 9 weeks), the inverted U-shaped dose–response curve became more pronounced and after 9 weeks exposure to 100 μgBPA/l, embryo production was significantly lower than in the controls ($P<0.05$).

3.3. Experiment three: effects of 17α-ethinylestradiol on vitellogenin synthesis and egg production in the fathead minnow, Pimephales promelas

The results presented for this experiment are part of a more comprehensive study that was recently published. (Pawlowski et al., in press.)

3.3.1. Mortality and growth

All of the fish survived the EE_2 treatment period and there were no subsequent mortalities during the reproduction performance studies. There were, however, effects of EE_2 on growth and condition factor (both were inhibited) in male fish at 10 ngEE_2/l-and in female fish at 100 ngEE_2/l, after 3 weeks of exposure.

3.3.2. Plasma vitellogenin

In control males, plasma VTG concentrations were less than 160 ng/ml. In males exposed to >0.1 ngEE_2/ml, there was a dose-dependent induction of plasma VTG, with a maximum plasma concentration of 77.500 ngVTG/ml at an exposure concentration of 100 ngEE_2/l (Fig. 3a) and, therefore, a 500-fold increase in VTG in exposed males relative to the controls. Control females had plasma VTG concentrations approximately 60-fold higher than in the control males, at around 10 μg/ml. As in the males, concentrations of EE_2 between 1 and 100 ng/l induced a dose-dependent induction of VTG. At an exposure concentration of 100 ngEE_2/l, plasma VTG concentrations in the females were 33-fold higher than in un-stimulated females.

3.3.3. Egg production

In control fathead minnows, the mean number of eggs spawned was 150 per pair/spawning during the 3 weeks following exposure. At concentrations of EE_2 ranging between 0.1 and 1 ng/l, there was a dose-dependent increase in the mean number of eggs spawned/pair, with the number almost doubling from 200+ to 390+, respectively (R^2=0.9271, P<0.05; Fig. 3b). At EE_2 concentrations greater than 1 ng/l, however, the number of spawned eggs per pair progressively decreased with increasing EE_2 dose, to less than 100 at 10 ngEE_2/l and a cessation of spawning occurred at 100 ng/l EE_2 (R^2=0.9814, P<0.001).

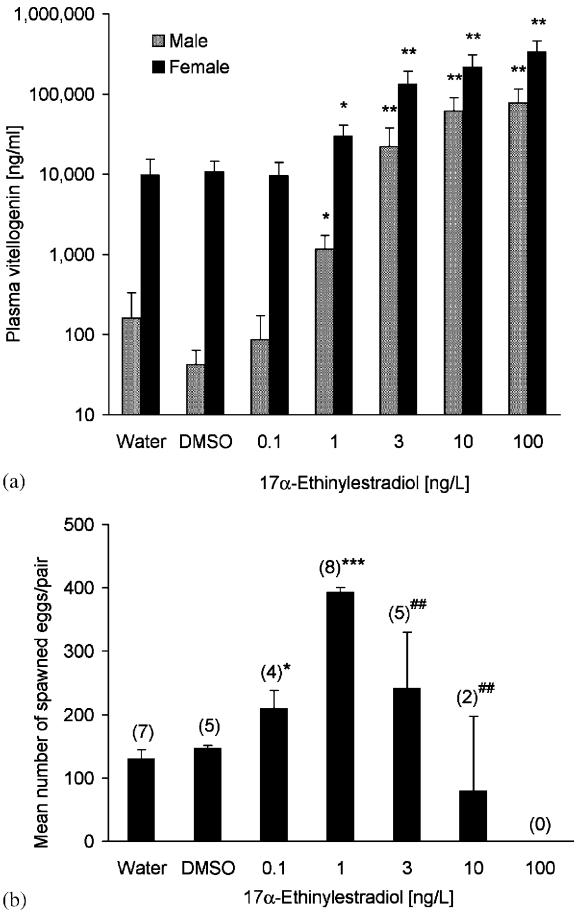

Fig. 3. *Pimephales promelas* (a) Plasma vitellogenin concentrations measured in male and female fathead minnows after 3 weeks exposure to 0, 0.1, 1 10 or 100 ng 17α-ethinylestradiol/l. (b) Egg production by spawning pairs of fathead minnows after 3 weeks exposure to 0, 0.1, 1, 10 or 100 ng 17α-ethinylestradiol/l. Means and standard errors are shown. Asterisks indicate statistical significant differences from the control *P<0.05, **P<0.01, ***P<0.001.

4. Discussion

The results of these experiments clearly demonstrate that estrogenic chemicals (and estrogenic effluents) that are known to cause reproductive effects in fish, also cause reproductive effects in the aquatic prosobranch snail, *P. antipodarum*. Both vitellogenin induction and egg production (in fish) were highly responsive to EE_2, and to the estrogenic effluent, as expected, with thresholds for effects similar to those reported in previous studies (for the same effluent: Harries et al., 1999; Rodgers-Gray et al., 2001; and for EE_2: Lange et al., 2001; Pawlowski et al., in press). Egg production in snails was also highly sensitive to estrogens and to estrogenic effluents; effective concentrations of EE_2 that induced altered reproductive output were of the same order of magnitude as occurred in the fathead minnow, *Pimaphales promelas* (in the low nanogram per litre range). Furthermore, the patterns

of the dose–response curves to estrogens (in terms of egg and embryo production) were similar in both the fathead minnow (*Pimaphales promelas)* and in *P. antipodarum*. Overall, the data indicate that the nature of the reproductive effects of estrogens, in this prosobranch snail, are not dissimilar to those that can occur in fish. These findings support the contention that egg production in prosobranch snails may potentially be used as a sensitive endpoint for testing estrogenic chemicals (Oehlmann et al., 2000), with relevance to vertebrates.

4.1. Comparative effects of estrogens in invertebrates

Effects of xenoestrogens on egg production have also been reported for other invertebrates. For example, the xenoestrogen BPA has been reported to induce egg production in the copepod *Acartia tonsa* (Andersen et al., 1999) and the polychaete worm *Dinophylus* (Depledge and Billinghurst, 1999), albeit that both of these species are much less sensitive to estrogen exposure than *P. antipodarum*. In most other invertebrate species, however, including some species of snail, (e.g. *Lymnaea stagnalis*; Czech et al., 2001), although estrogens are reported to be involved in sexual maturation and egg production, exposure to estrogens/xenestrogens has been found to have little, if any, effect on reproduction (Baldwin et al., 1998; Czech et al., 2001; Kahl et al., 1997; Meregalli et al., 2001; Woin and Brönmark, 1992; Zou and Fingerman, 1997), thus underlining the fact that the comparative responses of invertebrates to EDCs can differ widely, even within a taxon. Only in the arthropods, in which ecdysones (rather than estrogens) are known to control egg maturation and production, is there an established mechanism to explain why these animals are less sensitive to estrogens than are vertebrates (DeFur et al., 1999). In all other invertebrate taxa, little or nothing is known about their fundamental endocrinology and hence, the reasons for the comparability of the responses of fish and molluscs seen in this study can, at this stage, only be speculated.

In contrast to the invertebrates, in mammals and other vertebrates, estrogens have been known for many years to have a direct influence on folliculogenesis and cause both proliferative and differentiative effects on the somatic cells of the follicles, which contain estrogen receptors (Hillier, 2001). In most invertebrates, however, including prosobranch snails, almost nothing is known about the endocrinology underlying the egg-laying response. In the pulmonate snail, *Lymnaea stagnalis*, and in the opisthobranch, *Aplysia*, egg-laying is known to be stimulated by the ovulation inducing hormone CDCH (Caudodorsal cell hormone) produced by a group of neurones in the cerebral ganglia (Roubos and Vanheumen, 1994; Li et al., 1999). There is also evidence of a role for a neural GnRH-like peptide in regulating reproduction in freshwater snails (Young et al. 1999), as occurs in the brain of the vertebrates. Added to this, both estrogen and androgen concentrations in the tissues of prosobranch snails are known to vary with the seasonal reproductive cycle, although relationships between the neural factors and the steroids, and thus a vertebrate-like endocrine system, have not been demonstrated. Further work is necessary to explore the mechanisms underlying the egg-laying response in molluscs in order to understand how estrogens interact to alter embryo production.

4.2. Comparative sensitivities of fish to the estrogenic effluent

In contrast to the invertebrates, the sensitivities of vertebrates to environmental estrogens have been studied more widely and might be expected to vary widely given the relatively low level of conservation of the ligand binding domains of the estrogen receptors (e.g. Fielden et al., 1997 reported only 58% conservation in the ligand binding domain of ERα from several different vertebrates). In this study, response times for vitellogenin induction on exposure to estrogenic effluents were similar in the two divergent fish species (a salmonid and a cyprinid fish), occurring after 3 and 7 days exposure (to 50 and 100% effluent) and indicating similar sensitivities to the mixture of estrogenic chemicals. There was an indication that the vitellogenic response in rainbow trout may be a little more sensitive to estrogenic effluent, compared with the carp, because after 28 days exposure to the treated effluent, the critical threshold for VTG induction was 12.5% in the trout compared with 25% for the carp. Nevertheless, the relative sensitivities of the two fish species were highly comparable. This finding is in contrast with a previous study (Routledge et al., 1998), where exposure to the natural estrogen estradiol-17β, indicated that a salmonid fish (rainbow trout) was considerably

more sensitive than a cyprinid fish (roach). That study was, however, complicated by the fact that the different fish species used were at different stages of reproductive development and it is now well established that changes in estrogen (and other) receptor number and affinity change seasonally (e.g. Campbell et al., 1994). In this study, both fish species were at a similar stage of sexual immaturity, which excluded possible difficulties with the comparability of the responses due to differences in seasonal sexual status.

Despite their comparable sensitivities to estrogens, there were differences in the magnitude (up to tenfold) of the response between carp and trout. The higher response in trout compared with carp is likely to be linked with differences in their reproductive biology and/or related to the exposure conditions adopted. In the normal cycle of reproductive development, in maturing female rainbow trout, plasma VTG concentrations reach 50 mgVTG/ml, or more (Copeland et al., 1996), compared with only a few mgVTG/ml in female carp (Tyler et al., 1996) and thus the capacities of the systems for VTG synthesis may differ between the two species. Differences in the magnitude of the response, as a consequence of the exposure conditions, may be related to the temperature of the effluents in these studies; they ranged between 7 and 11 °C, and these are optimal for trout physiology, but sub-optimal for carp, which prefer slightly higher temperatures (15–20 °C).

4.3. Comparative sensitivities of Potamopyrgus and fish to estrogenic chemicals and effluent

In contrast with the vitellogenic response in fish, that occurred after only 3 days exposure to 100% effluent, alterations in embryo number in snails occurred after 14 days exposure to the same concentrations of effluent, indicating a slower effect of the effluent on embryo production in the snails than on vitellogenin induction in the trout and the carp. This latency in effect in snails compared with fish is not surprising, given the different nature of the responses studied: a single protein induction in fish versus embryo synthesis in snails. In the laboratory studies, embryo production in the snail was similarly responsive, as an endpoint for EE_2 exposure, as egg production in fish (compare Fig. 2aFig. 3b) over the 21 day study period. In both animals, there was an increased egg-laying (fish) and embryo production (snails) at low exposure concentrations of EE_2 (25 ng/l in the mollusc and 0.1–10 ng/l in the fish). Stimulatory effects of EE_2 on egg production occurred in fish up to an exposure dose of 3 ngEE_2/l followed by inhibitory effects at higher doses, culminating in a cessation of egg production at 100 ngEE_2/l. In snails, however, even at an exposure dose of 100 ng/l EE_2, there was an enhanced embryo production above the controls. This enhancement, however, was lower than that seen at 25 ngEE_2/l. Taken together, the results of the two separate experiments for EE_2 suggest that fathead minnows (*Pimaphales promelas)* are more sensitive to the reproductive effects of EE_2 compared with *Potamopyrgus*. It should be noted, however, that because of the nature of the system, the actual concentrations of test chemicals in semi-static exposures are often much lower (of the order of 20–50%) than the nominals, whereas in flow through exposures, the nominal and actual concentrations are generally more closely matched. No analytical chemistry was carried out on the exposure water, and hence a precise figure on the relative potency of EE_2 for effects on reproduction in the fathead minnow compared with *Potamopyrgus*, cannot be provided.

Concentrations of BPA and OP effecting embryo production in *Potamapyrgus* were at least two orders of magnitude higher than for effects seen with EE_2. These differences in relative potency for embryo production in snails are similar to those reported for vitellogenic and reproductive effects in fish (Ankley et al. 2001; Arcand-Hoy and Benson, 1998) and for other effects in other vertebrates. Furthermore, the relative potencies for interaction of the various xenestrogens (including BPA, OP and 4-*tert* nonylphenol) with the vertebrate estrogen receptor are similarly between two and three orders of magnitude lower than for ethinylestradiol.

In contrast to the higher sensitivity to EE_2 in fish versus snails, concentrations of BPA required to produce a reproductive response in snails appeared lower than the LOECs that have been previously reported for BPA in fish: In studies by Sohoni et al. (2001), exposure of fathead minnow to 1280 μgBPA/l for 164 days inhibited egg production whilst in *P. antipodarum*, effects on embryo production occurred at 1 μgBPA/l. The higher relative sensitivity of *Potamopyrgus* to the reproductive effects of BPA compared with fish (more than two orders of magnitude

difference; Sohoni et al., 2001) although surprising, is further supported by other studies in which Oehlmann et al. (2000) reported reproductive effects in the prosobranch snail *Marisa cornuarietis* at concentrations as low as 1 μgBPA/l. The findings in snails thus indicate that BPA might pose a significant risk to some aquatic invertebrates by affecting reproduction at environmentally relevant concentrations that do not appear to affect vertebrate animals. Notwithstanding this, it is also worth emphasising that in the whole life cycle study, by Sohoni et al. (2001), although reproductive effects in fathead minnows only occurred at very high exposure doses, there was an inhibition of spermatogenesis at exposure concentrations of 16 μg/l in the F0 generation and at 1 μgBPA/l in the F1 generation (the exposure period was 164 days). Further support for a higher possible sensitivity of snails to the effects of xenoestrogens may be derived from our effluent study, where after 14 days exposure, reproductive effects occurred at all effluent concentrations and in river water, compared with a LOEC in fish for VTG induction of 25% effluent for the same time point. The snail data indicates that the river water *might* contain a low level of estrogenic activity, although previous chemical analyses for steroid estrogens and nonylphenolic chemicals have not shown this (Kelly, personal communication). The detection limits for the analyses of steroid estrogens and nonylphenol in the effluent at that time however were only 0.5 ng/l (for EE_2) and 0.2 μg/l (for NP) and no consideration was given to the total estrogenic load (mixture) in the effluent. Overall, these data indicate that fish appear more sensitive than snails to disruption in reproductive output caused by EE_2, but that snails may be more responsive to low concentrations of some xenoestrogens (and/or estrogenic effluent) than fish. Further experimental work will be necessary to investigate the reasons for the apparent differences in sensitivities of the fish and snails to the same chemical.

Another similarity in the response of fish and the snail to the estrogenic effluent was the lowering of the critical threshold for a response with increased duration of exposure. This has been reported to occur in several other fish species after exposure to single chemicals or to estrogenic effluents (Rodgers-Gray et al., 2000; Sohoni et al., 2001). It is not known whether this effect occurs as a result of an enhanced sensitivity to estrogen with increased duration of exposure and/or, due to an accumulation of estrogenic chemicals in the target tissue.

4.4. The nature of dose–response curves for reproductive effects of estrogen in Potamopyrgus and fathead minnows

The dose–response curve for vitellogenin induction in fish exposed to EE_2 and the estrogenic effluent was classically sigmoidal in shape. In contrast, the reproductive output response curve in snails exposed to estrogenic effluents, EE_2, and xenoestrogens, or of fathead minnows exposed to EE_2, were more complex and of the inverted U-shaped type. In the snail, the inverted U-shaped response for reproductive output was consistent for the different sampling days for both the effluent and the individual estrogenic chemicals. Other studies have also reported inverted U-shaped dose–response curves notably for BPA (Giesy et al., 2000; egg production, plasma vitellogenin and plasma estradiol in fathead minnow; vomSaal et al., 1997; prostate weight in mice). Chemical hormesis, as this type of response is called, is low-dose stimulation followed by higher-dose inhibition (Calabrese and Baldwin, 1998) and is now recognised as a type of response to a number of chemicals (Davis and Svendsgaard, 1990, 1994). Unravelling the mechanisms of the interactions of estrogenic chemicals in fish and molluscs is required to develop a true understanding of how these estrogenic chemicals mediate this type of reproductive effect.

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