Environment Agency

Evidence

Investigations of endocrine disruption in UK freshwater molluscs

Report: SC030279/R1

Integrated catchment science programme

Evidence Directorate

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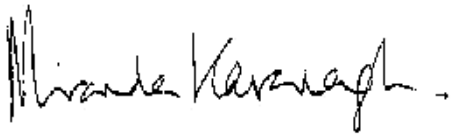

Miranda Kavanagh

Director of Evidence

Executive Summary

The association between the presence of domestic sewage effluent and abnormal reproductive development in fish, in British rivers, is widely recognised, but very little is known about the effects of exposure to domestic sewage effluent on invertebrates. This project was commissioned to begin to fill this knowledge gap. It concentrates on native freshwater gastropod molluscs, and has two major scientific aims:

1. To determine the likely existence and extent of endocrine disruption in UK native freshwater gastropods.
The natural oestrogen, 17β-estradiol (E2), or a mixture of endocrine active chemicals (EDCs) known to affect reproductive development in fish, were assessed for their effects on several species of gastropod mollusc under field conditions (outdoor tanks or 'mesocosms' supplied with river water). Growth, survival and reproduction were assessed in adult snails and their offspring.

2. To assess the suitability of several native gastropod mollusc species, with different reproductive strategies, as test organisms for endocrine disruptors.
This includes:

- investigating and developing methods for culturing selected snail species in the laboratory;
- assessing adult survival, growth and reproduction, and hatching success, growth and sexual development of offspring, under laboratory conditions;
- assessing the effects of exposure to 17β-estradiol on growth, reproduction and survival under laboratory conditions.

The project's key findings were that:

- **Adult reproduction in native pulmonate and prosobranch mollusc species may be affected by exposure to E2 under semi-field (mesocosm) conditions**.
  Maintaining chemical concentrations in the mesocosms was difficult. The differences in exposure, between treatments, were less than expected and typically the levels of added chemicals decreased throughout the exposure periods (possibly due to microbial activity and adsorption of the chemicals by macro algae). Despite this, differences were found in the levels of exposure between treatments and correlated differences in reproductive output of some snail species. The magnitude and direction of the reproductive response was influenced by the chemical dose, photoperiod and temperature. In the first mesocosm experiment, exposure of snails (*Planorbarius corneus*) to E2 resulted in an inverted U-shaped dose response curve, over the 12-week exposure period. In a subsequent experiment (using lower concentrations of E2), three gastropod mollusc species (*Planorbarius corneus*, *Viviparus viviparus* and *Bithynia tentaculata*) showed an increase in adult reproductive output in response to E2 exposure. Egg laying behaviour in *P. corneus* was prolonged beyond the normal autumnal decline, an effect seen previously in *P. corneus* exposed to treated domestic sewage effluent. In *V. viviparus* there was an increase in embryo production.

- **Early gonadal development may be most sensitive to the effects of E2 and EDCs.**
  In the first mesocosm experiment, exposure to increasing levels of E2 resulted in poorly differentiated gonads, intersexuality (both male and female gametes in the gonad) and changes in the sex ratio of surviving viviparid offspring (although the numbers collected were low). In a subsequent experiment, E2 exposure during early development disrupted gametogenesis in *P. corneus* (Sertoli cell disruption and a decrease in the activity of the vitellogenic area) and *V. viviparus*. Some of these effects (particularly on the oocytes) persisted after a year of depuration. A similar effect was seen in snails exposed to high concentrations of a mixture of EDCs.
- **Some snail species are more amenable to laboratory conditions than others.**
  The feasibility of rearing a range of freshwater molluscs in the laboratory, including *P. corneus* and *V. viviparus*, was studied. Of these two species, *P. corneus* (a pulmonate hermaphrodite egg layer) has shown the most promise. Other pulmonate species can be kept under the conditions described for *P. corneus*. In contrast, establishing laboratory populations of prosobranch species (*V. viviparus* and *B. tentaculata*) has proved more difficult. Consequently, it was not possible to establish stable populations of these species during this project.
- **Variability between individuals must be reduced if mollusc tests are to be developed.**
  Laboratory studies of reproductive output and hatching were carried out using *P. corneus*. There were considerable differences in: the number of eggs per egg mass, the cumulative numbers of egg masses and eggs, hatching success and survival amongst individuals. A method of dividing individual egg masses was shown to reduce the variation in hatching success between replicate treatments. This may be a useful approach when assessing exposure effects on development, under laboratory conditions. Some of the variability in reproductive output may be due to different levels of parasitism in individuals obtained from biological supply houses (caught from the wild). Therefore, studies to establish laboratory cultures of native molluscs should be undertaken.
- **Temperature is an important modulator of responses to chemical exposure in molluscs.**
  In the laboratory, E2 affects reproductive output in *P. Corneus*, but the effect of the chemical was strongly influenced by temperature. At low temperatures (15°C), E2 inhibited the natural decline in the rate of reproductive output that was seen in control *P. corneus* snails at this temperature. At a higher temperature (20°C), E2 appeared to have a slight inhibitory effect on reproductive output, compared to the controls. The effect of E2 on the seasonal reproductive response of *P. corneus* was also observed in the mesocosm experiments, as a prolongation of egg laying late into autumn. Therefore, the design of mollusc tests that use seasonal native species must take into account the role of temperature, and changes in temperature, on biological responses.
- **Developmental exposure to mixtures of estrogenic chemicals may result in a predisposition to parasite infection.**
  These results provide preliminary data that shows that developmental exposure to EDCs increases the likelihood of parasitism, possibly due to suppression of the immune system. The association between parasitism and exposure to chemicals was found by histological analysis of tissue sections and this highlights the potential of histological methods when investigating the effects of chemicals on wildlife.

Technical Summary

The association between the presence of domestic sewage effluent and abnormal reproductive development in fish, in British rivers, is widely recognised, but there is relatively little known about the potential effects of exposure to domestic sewage effluent on native freshwater gastropod molluscs. The need to address this issue came to light when laboratory studies on a tropical species of freshwater gastropod mollusc (*Marisa cornuarietis*) suggested this species was sensitive to low levels of endocrine disrupting chemicals (hormone mimics) known to be present in sewage effluents.

A previous study, in outdoor tanks, on two species of native gastropod mollusc exposed to sewage effluents known to contain a mixture of endocrine active chemicals, found that effluents could have dose dependent effects on reproduction and sexual development in some molluscs (Clarke et al. 2009). However, it was unclear whether it was chemicals in the effluents (endocrine disrupting or otherwise) that were causing these effects. Therefore, the first priority of this study was to assess the effects of endocrine disrupting chemicals (EDCs), known to be present in the sewage effluents, on reproduction in the same species of snails and under the same field conditions.

The two species selected represent the two main groups of European freshwater snails. One of these, the pulmonate snail *Planorbarius corneus*, is a simultaneous hermaphrodite i.e. it produces both male and female sex cells in the same gonad. The other species, the prosobranch snail *Viviparus viviparus*, has separate sexes and gives birth to live young. Some preliminary treatments also used another prosobranch species (*Bithynia tentaculata*), which is an egg layer.

If these snails are sensitive to EDCs, as are fish, then freshwater gastropod molluscs could perhaps be used in chemical testing strategies, as surrogates for vertebrates. Thus, a second priority for this project was to explore the feasibility of developing laboratory tests for endocrine disruptors using gastropod molluscs by (a) identifying easily cultured species and (b) identifying species sensitive to a range of known endocrine disruptors and characterising their response to ECDs under laboratory test conditions.

Key findings are:

<u>Objective 1: Exposure of native molluscs, in outdoor mesocosms, to 17β-estradiol (E2) or a mixture of EDCs. Effects on both adults and developmentally-exposed offspring.</u>

It was difficult to maintain a consistent dose of steroids and other EDCs (including alkyphenols, alkylphenol ethoxylates and BPA) in the outdoor mesocosm tanks. The measured concentrations of chemicals in the tanks varied depending on water temperature, the amount of macrophyte growth in the tanks and the unpredictable nature of the river water used as a diluent. A river water only (control) tank was used and there was a gradient of dosed chemicals in the tanks from low (in the reference tank) to high (in the highest dosed tanks).

<u>17β-Estradiol studies</u>

The mean reproduction rate in adult *P. corneus* appeared to be affected by exposure to E2. However, there was a high degree of variability between replicate groups of snails and the observed effects were not statistically significant at the 95% level in all cases. In *P. corneus*, exposure to a high E2 dose (HE2) in Experiment 1 (48.1ng/L) and

Experiment 2 (70.3ng/L) resulted in snails ceasing to lay eggs after 6 and 8 weeks of exposure, respectively. In Experiment 2, exposure to a low E2 dose (LE2; a nominal dose of 10ng/L) caused a prolongation in egg laying. This extended through the period when a normal seasonal decline and cessation in egg laying occurred in the control groups.

In *V. viviparus*, the number of unhatched embryos found in the brood pouches of snails exposed to LE2, was double that of the control females (statistically significant with 99% confidence). In preliminary studies using *B. tentaculata*, the productivity of the control group declined steadily over the 16 week exposure, but in snails exposed to HE2 there was an increase in the rate of egg production from week 10 onwards, relative to the control group. This difference was statistically significant (99% confidence) at week 14. However, there was a very high level of variation, between replicates, in the number of eggs laid; much of this was attributed to differing proportions of males and females in each replicate. Interpretation of these results was further complicated because some replicates were removed from the analysis when they were found to be parasitized and approximately 25% of the 96 individual test organisms per treatment escaped over the duration of the treatment.

In the F1 generation of *V. viviparus*, there was a dose-dependent increase in the male:female sex ratio (statistically significant, >99.9% confidence). Moreover, in Experiment 1, intersex snails (males with female brood pouches and snails with both male and female gametes in the gonads) were found (5% in low E2 and 14% in high E2) while none were found in the control tank. Developmental exposure of F1 *P.corneus* to E2 resulted in a significant delay in egg laying, leading to a reduction in the mean number of egg masses laid per snail over the 14 weeks of the study.

In an effort to find robust measures of reproductive development and reproduction, male and female indices were developed that describe the appearance of the gonad tissues after histological preparation. These proved to be very useful and quantifiable measures of the effects of exposure. The effects of the treatments on the offspring of exposed snails were more obvious than the effects seen in their parents. In *P. corneus*, for example, abnormal sloughing of the germ cell support cells (Sertoli cells) and immature germ cells, into the lumen of the gonad, was seen in the HE2 exposure groups. In both the HE2 and LE2 groups there was a decrease in the area of the gonad that was composed of mature reproductive tissue (statistically significant with greater than 99.9% confidence). Some of these effects (particularly on the developing eggs) persisted even after a year depuration. In *V. viviparus*, no obvious differences could be seen in the exposed adults, but the number, and rate of development, of sperm cells and developing eggs in the F1 males and females decreased in a dose-dependent manner (statistically significant, >99.9 % confidence).

Mixtures study

Exposure to a mixture of endocrine disrupting chemicals (including estradiol, oestrone, ethinylestradiol, nonylphenol, octylphenol and bisphenol-A) did not cause high mortality in either P.corneus or V.viviparus, at any of the concentrations tested. Survival of adult snails was similar in all exposure groups until week 10 (there were >30 snails in all treatment groups) after which the River Water with solvent only (RW+S) mesocosm tank suffered very high mortalities. The collapse of the groups in the RW+S tank is unlikely to have been due to the presence of ethanol. The solvent concentration in the dosed tanks (approximately 0.005□g/L) was much lower than the solvent concentrations (100□g/L) reported, by the OECD, to have effects on reproduction of aquatic organisms. This figure has been revised downwards, to 20□g/L, by Hutchinson et al. (2006).

In the offspring of *V.viviparus* there was evidence of sex specific effects on growth. In male offspring, the group exposed to the highest concentration of mixture (HM) were significantly smaller (in terms of shell length and weight) than individuals in the groups exposed to RW, RW+S and the lower concentration of the mixture (LM). In the female offspring, the HM female offspring were smaller than the RW group, but the RW+S snails were also very small and so no conclusions could be drawn. Survival over winter was highest in the HM group (75%) compared to RW (53%) and LM (33%). However, population density may have been a confounding factor in overwinter survival; only 84 snails were left to overwinter in the HM tank, compared with 520 in the RW and 200 in the LM tanks. Among the offspring of *P. corneus*, snails exposed to RW were larger and heavier than those from the LM or HM groups (statistically significant differences).

In this experiment histopathological measures were used to assess male and female sexual development. A dose-dependent decrease in activity in the vitellogenic area (developing eggs with their supporting cells and/or sperm with their supporting cells) was found in adult *P. corneus* snails and their offspring. This reduction was not statistically significant in the adults. In the offspring, the large differences between the HM and the RW+S and RW treatments were significant at the 95% and 99.9% confidence levels, respectively. These differences persisted until after these snails had reproduced. Large differences were seen in terms of the area of the adult gonad that was covered with maturing germ cells when comparing the HM group and the non-exposed groups. These differences were seen in both adults and their offspring. The differences were statistically significant, >99% confidence, but opposite effects were seen in the adults compared to the offspring. In adult *P. corneus*, as with the E2 exposures, a greater degree of sloughing of the Sertoli cells (supporting the germ cells) and the germ cells themselves was seen in the HM and LM exposure groups, compared to the RW exposed snails (the differences were not statistically significant). The percentages of different oocyte developmental stages, present in the gonad, were significantly different (with greater than 95% confidence) between the exposed and non exposed groups. The percentage of early stage oocytes (stages two and three) was highest in snails exposed to LM, while the percentage of mature oocytes (stage four) was highest in snails exposed to RW+S. These results were partially compromised by the lower survival rate of snails in the solvent tank. However, in the F1 generation, larger and more significant differences could be seen between exposed and non exposed snails. Both LM and HM exposure groups had higher percentages of early stage oocytes (stage 1) compared with the controls (statistically significant, >95% confidence).

In adult female *V. viviparus*, large differences in the number of oocytes per section were observed between RW exposed (11.0 oocytes per section) and LM groups (4.2 oocytes per section). These differences were statistically significant (>95% confidence). In the F1 generation, both the HM and LM groups had significantly fewer oocytes per snail than in the controls. This appeared to be a dose-dependent effect, although the absolute number of oocytes per section was much lower than in the adults. There were no effects on spermatogenesis in adult male *V. viviparus*. In the male offspring the spermatogenesis score was highest in snails from the control and LM tanks and was lowest in HM snails (statistically significant, >99% confidence). However, when these snails had depurated there were no significant differences between the groups.

Trematode parasites were found in both *P. corneus* and *V. viviparus*. However, in *P.corneus* only one snail in the adult exposure was affected and no snails were affected in the F1 generation. In *V. viviparus*, the majority of adult snails had some level of parasitism, but in the offspring parasitism was found only in the dosed tanks and not in either of the control tanks. The severity of parasite infection increased with

dose in both male and female snails (statistically significant, 99% confidence). Parasitism did not appear to be correlated with the degree of sexual maturity or with reproductive output.

Objective 2a: Culture

Some snail species are more amenable to being cultured under laboratory conditions than others. The feasibility of rearing a range of freshwater molluscs in the laboratory, including *P. corneus* and *V. viviparus* has been investigated. Of these two species, *P. corneus* (a pulmonate hermaphrodite egg layer) has shown the most promise. Other pulmonate species can be kept under the conditions developed for this species. In contrast, establishing laboratory populations of prosobranch species (*V. viviparus* and *B. tentaculata*) has proved more difficult and were not successful in establishing long term populations during this project. Of these two species only adults of *B. tentaculata* could be kept for periods of up to six months in the laboratory and laid egg masses consistently. However, their young did not survive beyond a certain age and so a continuous culture could not be developed.

Objective 2b: Laboratory Exposures.

Groups of snails were held under simulated springtime temperature and day-length (15^{o}C and 12 hours light/12 hours dark) or simulated summertime temperature and day-length (20^{o}C and 16 hours light/8 hours dark). Their reproductive output was monitored over a 4-week baseline period, after which some groups were exposed to 1, 10 or 100ng/l (nominal) E2 for a further 4-week period. The half life of E2 in the semi-static system decreased with increasing concentration of E2.

There was a high level of variability in the reproductive rates between the groups of snails, both before exposure started and during the exposures. It was apparent that most of the variation in egg mass production was caused by an unknown factor at the individual level. At the end of the experiment, when the snails were dissected, a low number of individuals were found to be heavily parasitised by diageneans in the reproductive tract (6.3% at 15^{o}C and 6.7% at 20^{o}C). These adults were considered to be virtually reproductively inactive and the data was adjusted to discount these parasitised individuals.

Egg production at high temperature was approximately double (700 eggs/adult) the rate at low temperature (360 eggs per adult). There was little evidence of any effect of E2 on egg production in the simulated summer conditions. In contrast, in simulated autumn conditions, the unexposed snails showed a significant reduction in their reproductive output, while there was little evidence of a decline in reproductive output from snails exposed to 100ng/l E2 (nominal).

A preliminary pair-breeding experiment was performed using *B. tentaculata* exposed to the same nominal concentrations of E2 for 18 weeks. Recovery of E2 from freshly dosed medium was satisfactory (between 78.4 and 86.4% of nominal). As with the *P. corneus* exposures, there was appreciable degradation of E2 over 48 hours. This continued over the course of the exposure so that only the highest concentration (100ng/L nominal) treatment was statistically distinguishable from the control by the end.

Of the 200 pairs in the experiment, only 21 pairs laid viable eggs. This low viability, in terms of egg productivity, could have been due to: incompatibility of the pairs (a drawback of pair-breeding studies, where a choice of mate is necessarily denied to the individuals), unsuitability of the test medium or housing of the organisms, or more likely, the high levels of parasitism that were observed in the population.

Conclusions

These studies highlight a broad range of issues that relate to the effects of oestrogens on snails. There is some evidence that exposure to E2 affects reproduction in all the species tested in outdoor mesocosms. However, interpretation of the mesocosm results is confounded by the difficulty of reliably dosing the chemical in a 'real world' scenario i.e. where bacterial degradation, sorption to sediments and algal growth affect the concentration of chemical in the water.

In the laboratory, similar effects on reproduction were observed, although these were less clear and varied with temperature. E2 can be inhibitory or stimulatory, depending on the dose, environmental conditions (seasonality) and degree of toxicity. Importantly, these results indicate that responses are most likely to be observed at low, or changing, temperatures than during constant warm conditions.

Histological analysis of the gonads was a useful technique for demonstrating the effects of chemicals on gastropod reproduction. Gametogenesis was disrupted in both the male and female parts of the gonad. However, disruption of the vitellogenic area of the gonad (i.e. oocyte development) was far more persistent than effects on spermatogenesis, in both *P. corneus* and *V. viviparus*.

Histological analysis also demonstrated that parasitism is common in molluscs collected from the wild. Many parasites can, directly or indirectly, affect reproduction so it is possible that much of the variability in reproductive output may reflect variation in the frequency and intensity of parasitism between groups of snails. The results indicate that chemical exposure may increase the likelihood of parasitism, possibly due to suppression of the immune system. In order to reduce the uncontrolled variability in reproductive outputs from molluscs used in laboratory tests, it will be necessary to develop parasite free laboratory strains of snails. .

The focus of this research has been on adult exposures to estrogenic chemicals, but there are indications that early stages in the life cycle are even more susceptible to adverse effects than adults. Therefore, further research on the effects of developmental exposures in molluscs should be a priority.

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Contents

Table of Figures

Table of Tables

1. Introduction

In UK freshwaters the association between endocrine disruptors, present in treated sewage effluents, and abnormal reproductive development in coarse fish is well established (Jobling et al. 1998). However, very little is known about whether these chemicals cause similar effects in invertebrates, as relatively little work has been conducted in this area. Some laboratory studies suggest that gastropod mollusc species are sensitive to the effects of environmental estrogens. In a subtropical gastropod mollusc, *Marisa cornuarietis*, exposure to the xenoestrogen bisphenol-A resulted in over-production of eggs, this led to blocked and ruptured oviducts, and death of the molluscs (Oehlmann et al. 2000). In the live-bearing species *Potamopyrgus antipodarum* (an introduced species of snail found throughout the UK and Europe), embryo production reportedly increased in the presence of very low concentrations of some estrogenic chemicals and decreased at higher concentrations of the same chemicals (Jobling et al. 2003). Known androgenic chemicals have been reported to masculinise *Marisa cornuarietis*, while anti-androgens reverse the effect (Tillmann et al. 2001).

In view of the huge diversity of Mollusca in the UK (there are 72 freshwater and brackish species) and the variety of their reproductive strategies, it is important to investigate the extent and likely severity of endocrine disruption in a range of UK mollusc species. Recent studies of the effects of sewage effluents containing EDCs on three gastropod mollusc species, suggest that effluents can cause dose-dependent effects on reproduction and sexual development in some native molluscs (Clarke et al. 2009). However, it is unclear which chemicals in effluents (endocrine disrupting or otherwise) are causing these effects. This study addresses these questions and contributes to an understanding of the significance, for other aquatic organisms, of the endocrine disruption effects that have been found in fish. The results will inform the setting of targets for measuring biological effects and responses. Techniques for assaying and monitoring the effects of EDCs on invertebrate species need to be developed to complement, or replace, those used for vertebrate species.

1.1 Objectives

1. To investigate, under field conditions (in tanks supplied with river water), the existence and likely extent of endocrine disruption in UK native freshwater gastropods. The study will assess the effects of EDCs, known to be present in sewage effluents and rivers, on reproduction and development in several species of gastropod mollusc that have different reproductive strategies.

2. To assess the suitability of a variety of gastropod mollusc species, with different reproductive strategies, as test organisms for the presence and effect of endocrine disruptors. This includes;
   a) exploring the feasibility of culturing selected EDC-sensitive snail species in the lab,
   b) assessing the effects, on snail reproduction, of exposure to selected EDCs in the laboratory,
   c) assessing the repeatability and robustness of mollusc tests as prospective tools for monitoring for endocrine disruption.

1.2 Scope

This study was restricted to a selection of mollusc species whose reproductive strategies allow them to be maintained in outdoor mesocosms. In the laboratory, the scope of the study was further restricted to species that could, potentially, be cultured in the laboratory (it was recognised that there was not enough time to establish large scale laboratory cultures for this work). The chemicals chosen for use in the exposure treatments were restricted to known vertebrate endocrine disruptors.

1.3 Approach

The exposures in the field mesocosm system were complemented, whenever possible, by controlled laboratory exposures. Laboratory exposures were designed to factor in aspects of seasonality. In both cases, measurements of reproductive output, hatchability (if appropriate) and morphological/histological characteristics were used.

2. Objective 1. Mesocosm studies

2.1 Mesocosm experiment 1a: the exposure of two UK native freshwater gastropod species to 17β-estradiol (E2) in an outdoor mesocosm

2.1.1 Methods

Experimental set up

The snails were exposed to high or low concentrations of E2 or to the river water alone, in three large outdoor tanks. These mesocosm tanks were situated at Langford Water Treatment Works (WTW), Essex. Langford WTW is part of Essex and Suffolk Water. It is in close proximity to both the River Blackwater and the River Chelmer (Figure 1). The mesocosms were 1m^3 tanks (Figure 2) fed with water from the River Blackwater abstracted at grid reference point TL835090.

Figure 1. Ordinance Survey Map of Langford village, Essex. The Essex and Suffolk water treatment works (labelled Wks) and reservoirs are shown at the centre of the map. This is where the mesocosm tanks are housed. They can be fed with river water from either the River Chelmer (labelled, running west to south-east), or The River Blackwater (not labelled, running north-west to south-east).

Each tank had a working volume of 1000 litres and a surface area of approximately 1m^2. Before entering the tanks, the river water was passed through activated carbon filters to remove trace organic compounds. The flow rate to the tanks was approximately 6l/min and the tank retention time was approximately 2 hours. The snails were housed in twelve cylindrical mesh sleeves (20cm in diameter) in each of the tanks. The sleeves were suspended from rods, laid horizontally across the top of each tank, and were submerged in the water to a depth of 50cm. Crossed glass

plates, 50cm long with four arms each 10cm wide, were inserted into each sleeve. These served the dual purpose of both holding the sleeves in shape and providing a surface on which algae and biofilm could grow. Each mesocosm tank was covered with a wire mesh to prevent interference from birds.

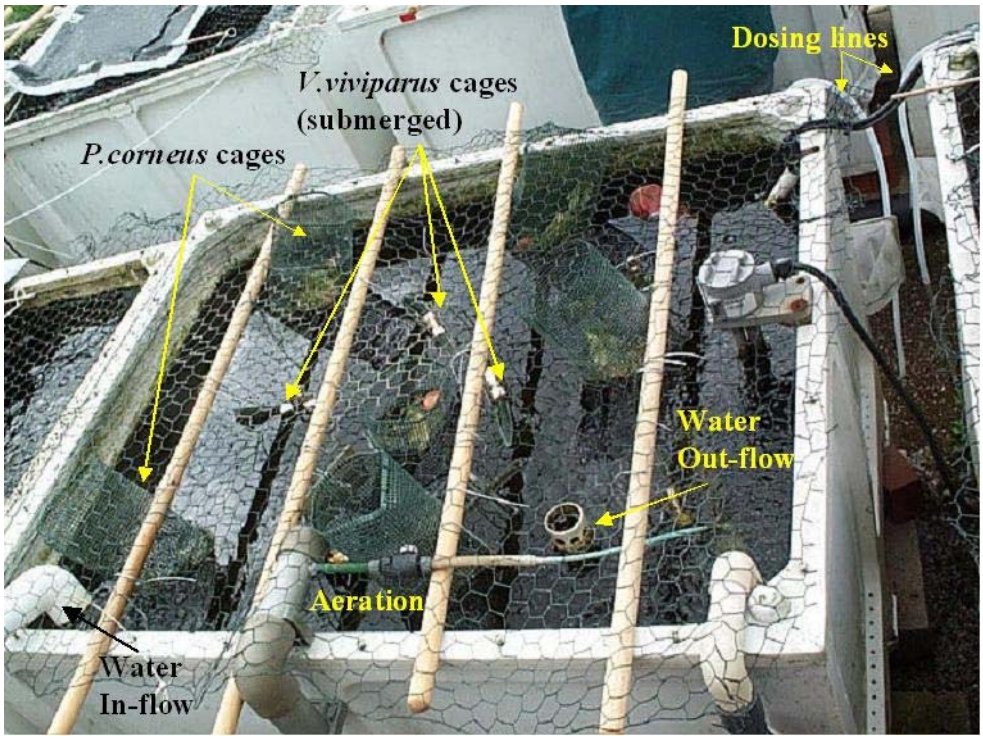

Figure 2. Photograph of a mesocosm tank. Cages for adult snails are suspended by wooden broom handles. *P. corneus* cages are positioned so that the (air breathing) snails can reach the surface, but not escape. *V. viviparus* cages are submerged, providing the best habitat possible for this deeper water (gilled) species. River water flows into the tank through the pipe at bottom left and out through the pipe at bottom middle. The chicken wire cover prevented predation by birds.

The two treatment tanks were dosed with 17β-estradiol (E2; Sigma, Poole) to nominal concentrations of 20 and 200ng/l. A stock solution of 1g/l E2 in ethanol was prepared. This was stored at 4°C in the dark and replaced every month. The stock solution was diluted into mixing tanks containing standing river water prior to dosing. The mixing tanks had a volume of 12l and had to be manually refilled every 48 hours. A peristaltic pump was used to continually dose the treatment tanks, from the mixing tanks, to give the appropriate nominal concentrations.

Flow rates to the tanks were checked daily and the pump rate checked fortnightly. On exiting the system, the water from all three tanks passed through an activated carbon filter to remove the dosed chemical. The filtered water was returned to the River Blackwater, downstream of the intake. The efficiency of carbon filtration in stripping steroid estrogens and other organics from contaminated water is well established (Choi, 2004; Knappe, 1999; Snyder, 2007; Ternes, 2002; Yu, 2009).

Monthly samples were taken from each tank. These were analysed for 17β-estradiol (E2), oestrone (E1) and 17α-ethinylestradiol (EE2) by the Environment Agency National Laboratory Service, Nottingham (NLS). The samples were collected in a 10l stainless steel bucket, homogenised and placed in clear glass Winchester bottles for shipment to the NLS on the same day. Samples were extracted within 36 hours of sampling. The samples were spiked with deuterated estrogen analogues, as internal

standard, prior to extraction on a styrene divinyl benzene polymer SPE cartridge. Steroids were then de-absorbed with DCM. The DCM extracts were concentrated and cleaned-up using gel permeation chromatography fractionation, followed by solvent exchange to 95/5 v/v isohexane/propan-2-ol and then normal phase chromatography in an amino LC column. The extracts were then evaporated to incipient dryness, dissolved in methanol and analysed using HPLC with negative ion atmospheric photo-ionisation interface and MS time-of-flight detection.

Test Organisms

The gastropod species selected for this study were the pulmonate *Planorbarius corneus* and the prosobranch *Viviparus viviparus.* The main reasons for these choices were:

- both snails are relatively large (length of longest axis generally >20mm), this makes for ease of handling and reduces escapes under field conditions;
- both are known (Clarke et al. 2009) to survive well in the conditions provided by this particular mesocosm system. Three other species were tried in previous studies, but did not thrive.

These two species have very different reproductive strategies. *P. corneus* is a simultaneous hermaphrodite that lays its eggs in a discreet flat, disc-like mass that has a tough outer coating (Costil and Daguzan, 1995b). In contrast, *V. viviparus* has two distinct sexes and the female is a live bearer. The embryos are brooded within the female until they are sufficiently developed to be fully independent at birth (Griffond, 1978). *P. corneus* were obtained from 'Blades' Biological Supply (originally captured from wild stocks in the River Rife, near Worthing). *V. viviparus* were collected from the Grand Union Canal in Cowley, at grid reference TQ055810.

All sexually mature animals (>15mm) were held for a few days in tanks of carbon filtered drinking water at Brunel University. They were then transported to the Langford WTW site in groups of approximately 50 in sealed plastic bags containing about 1l of water and 3l of air. The animals were weighed, measured along the longest axis and randomly placed in one of six sleeves in each mesocosm tank. Nine (±1) individuals of *P. corneus*, or 11 *V. viviparus* (approximately 2 males and 9 females), were allocated to each sleeve. The *P. corneus* were fed three times per week with about 10 leaves of organic lettuce and 0.4g of fish flakes (Tetramin). The *V. viviparus* were allowed to feed on the algae growing on the glass plates and in the water.

Sampling

P. corneus egg masses and *V. viviparus* juveniles were sampled and counted at 2 week intervals (Clarke et al. 2009). Egg masses were photographed and the number of eggs in each mass counted. Eggs were counted for a total of 14 weeks (2 weeks pre-exposure and 12 weeks of exposure). The total weight of egg masses from each of the replicate sleeves was recorded. At the end of the exposure period, all the adult animals were placed in a 5% solution of magnesium chloride for at least two hours to relax the tissues. The snails were then weighed and measured along the longest axis. The embryos were then removed and the remaining tissues were re-weighed before the gonad was removed and preserved.

2.1.2 Results

Chemical analysis

The mean recovery of E2 in river water was 87.7 ± 2.9%, with an uncertainty estimate in river water of 29.2%. Minimum reportable values (MRV) were 0.3ng/l (E2), 1.0ng/l (E1) and 0.1ng/l (EE2). Table 1 shows the results of the analysis for steroid estrogens. The concentrations of E2 in the river water in June and July, as reported by the NLS, were higher than expected, based on previous measurements from the Chelmer. Therefore, a sample was taken on the 3rd August that was sent to CEFAS to have these levels independently verified (see Table 2). The presence of E2 and E1 in this sample was independently confirmed by CEFAS. The next two samples (taken on the 17th August and 13th September) were analysed by both laboratories (Tables 1 and 2) to further corroborate the finding of E2 and E1 in the river water. CEFAS also detected the presence of EE2 on the last two sampling occasions. Concentrations of steroids at the outfall (after stripping and before discharge to the environment) were below quantification limits (data not shown).

Table 1. Levels of steroid estrogens (E2, E1 and EE2) measured by the Environment Agency National Laboratory Service, Nottingham. ND = not detected.

Date of Sampling	**E2 (ng/l)**			**E1 (ng/l)**			**EE2 (ng/l)**		
Nominal E2 for tank (ng/l)	*0*	*20*	*200*	*0*	*20*	*200*	*0*	*20*	*200*
14-06-04	6.14	89.9	104	2.44	39.5	67.3	ND	ND	ND
20-07-04	14.9	23.1	259	3.40	3.56	52.0	ND	ND	ND
17-08-04	10.0	51.2	228	6.81	14.1	70.6	ND	ND	ND
13-09-04	20.3	28.1	889	<1.0	<1.0	242	ND	ND	ND
Mean	12.8	48.1	370	4.22	19.1	108	ND	ND	ND
Standard Deviation	6.13	30.5	352	2.30	18.5	90.0	ND	ND	ND

Table 2. Levels of steroid estrogens (E2, E1 and EE2) measured by CEFAS. ND = not detected.

Date of Sampling	**E2 (ng/l)**			**E1 (ng/l)**			**EE2 (ng/l)**		
Nominal E2 for tank (ng/l)	0	20	200	0	20	200	0	20	200
03-08-04	3.7	16	121	2.6	3	32	<0.5	<0.5	<0.5
17-08-04	5.2	53	304	1.5	5.5	36	0.9	<0.5	<0.5
13-09-04	9.2	14	1120	3.1	2.2	468	0.7	<0.5	<0.5
Mean	6.03	27.67	515	2.4	3.57	178.66	0.8	ND	ND
Standard Deviation	2.84	21.96	531.8	0.82	1.72	250.57	0.14	ND	ND

Effects of E2 exposure on adult *P.corneus* growth, survival and reproduction

Mortality

In *P. corneus*, the groups exposed to river water (RW) and low E2 (LE2) had mortality rates of 10-15% by week 8, but the remaining animals then survived until the end of the treatment. There were higher mortality rates in the high E2 (HE2) group; over 40% mortality by the end of the exposure (Figure 3). This tank had significantly higher mortality than the RW tank from week 6 (ANOVA, P<0.05 on all sampling occasions).

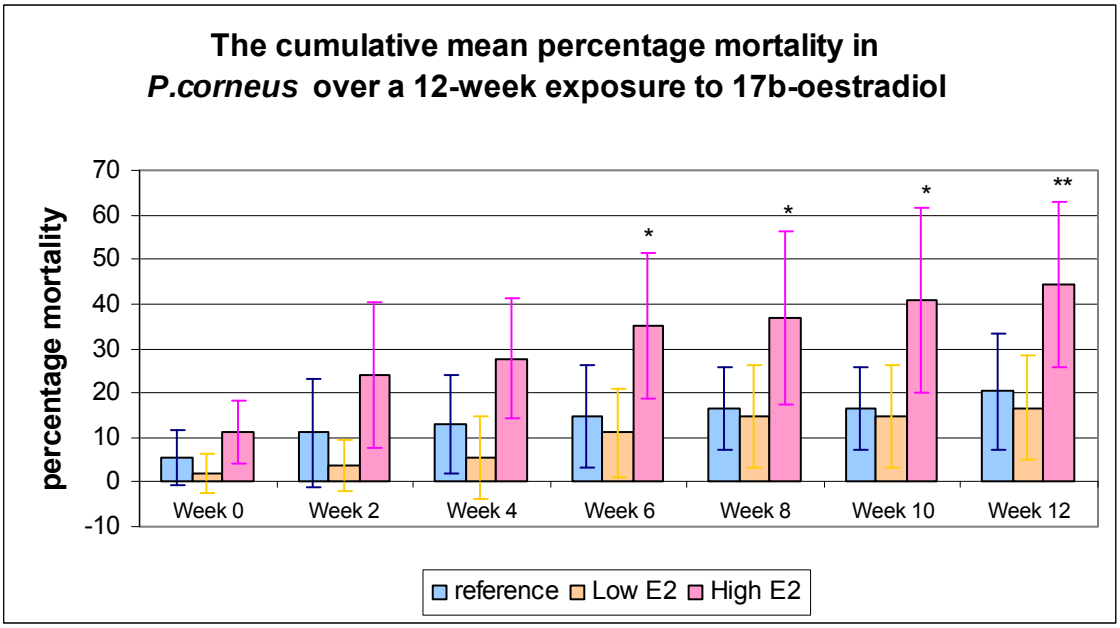

Figure 3. Mortality of *P. corneus* over 12-week E2 exposure. * indicates significantly different from river water exposure. Mean measured concentrations of E2 were 12.8ng/l in RW, 48.1ng/l in LE2 and 370ng/l in HE2.

Growth

All snails grew during the 12-week exposure period. The mean increase in shell diameter was 10-20% and the mean increase in weight was 40-60%, in all three tanks (Figure 4). There was a statistically significant difference (ANOVA, P<0.05) in length (but not weight) between the LE2 and RW treatments at the end of the experiment.

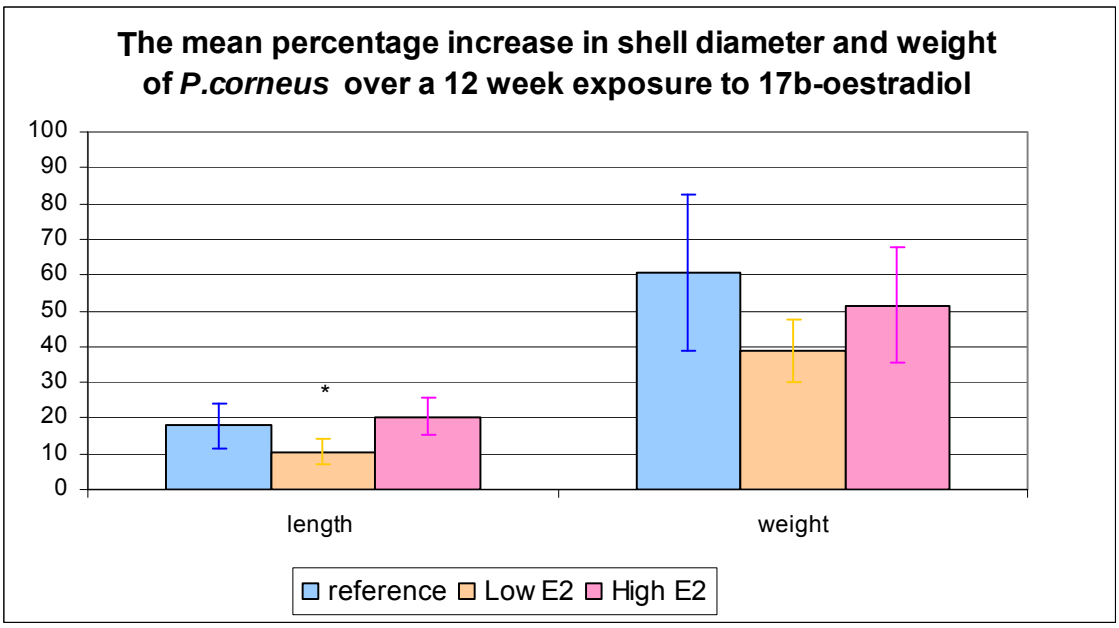

Figure 4. Percentage increase in *P. corneus* length and weight during 12-week exposure to E2. * indicates a statistically significant difference from river water exposure (P<0.05). There were 54 snails per treatment at the start of the experiment. Data based on 32 surviving snails in HE2, 46 in LE2 and 45 in RW at end of experiment.

Reproduction

At the first sampling point, 2 weeks into the exposure, the number of eggs laid per surviving snail was significantly reduced in both of the E2 dosed tanks, compared to the RW tank (ANOVA, P<0.01). Two weeks later (week 4) the reduction in the number of egg masses laid was more pronounced. By week 6 the snails treated with LE2 had virtually stopped laying egg masses. They recovered slightly at the next sampling point, but still laid less than one egg mass per surviving snail per week. Over the same period (weeks 4 to 8) the snails in the HE2 and the RW tanks remained close to constant, but the number of egg masses laid by the snails in the RW tank was falling at each sampling point so that, by week 8, the productivity from these two tanks was statistically indistinguishable. For the final 4 weeks of the exposure, all snails, in all tanks, laid less than 0.5 egg masses per week, on average (see Figure 5).

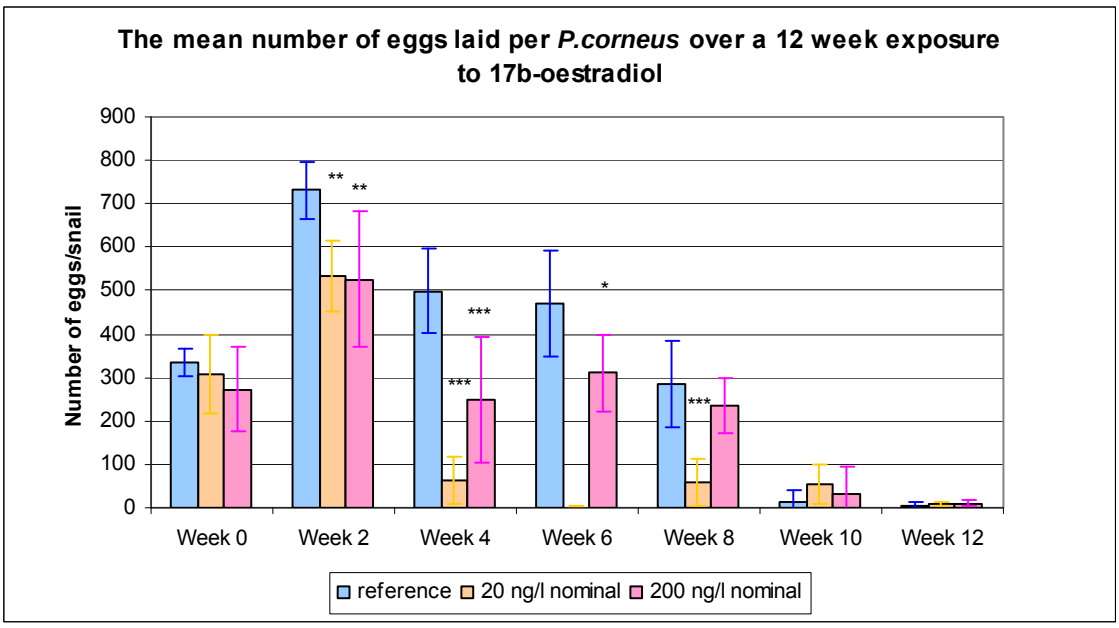

Figure 5. The mean number of eggs produced by surviving adult *P. corneus* at each sampling point during a 12-week exposure to E2. * indicates a statistically significant difference from RW where *p<0.05; ** p<0.01; *** p<0.001. Error bars denote standard deviation from the mean.

Effects of E2 exposure on adult *V.viviparus* growth, survival and reproduction.

Mortality

In this species there were high levels of mortality over the 12-week exposure, but most of the deaths occurred in the second half of the experiment. Until the sampling point at week 6, mortality in all three tanks remained below an average of 10%. After this, the snails in the control tank showed steadily increasing mortality, culminating in an average mortality per sleeve of almost 35%. The same pattern was observed in the LE2 and HE2 tanks, where mortality reached approximately 40% and 60%, respectively, at the end of the exposure (Figure 10). There appeared to be a dose-response relationship at most of the sampling points, but this apparent trend was not supported by the statistical analysis; mortality in the HE2 dose was only significantly greater than the control at week 6 (ANOVA, P<0.05).

Sex ratio

At the end of the exposure, when the remaining adults were sacrificed and dissected for sexual identification, there was some indication that more males than females had died with increasing E2 dose. Initially, all tanks had a higher proportion of females than males (9 females and 2 males per group, or ~82% female). At the end of the exposure there was no significant difference (ANOVA, P>0.05) in the proportion of male to female snails across treatments (on average 83.2% were female in RW, 88.3% in the LE2 treatment and 95.8% in the HE2 treatment).

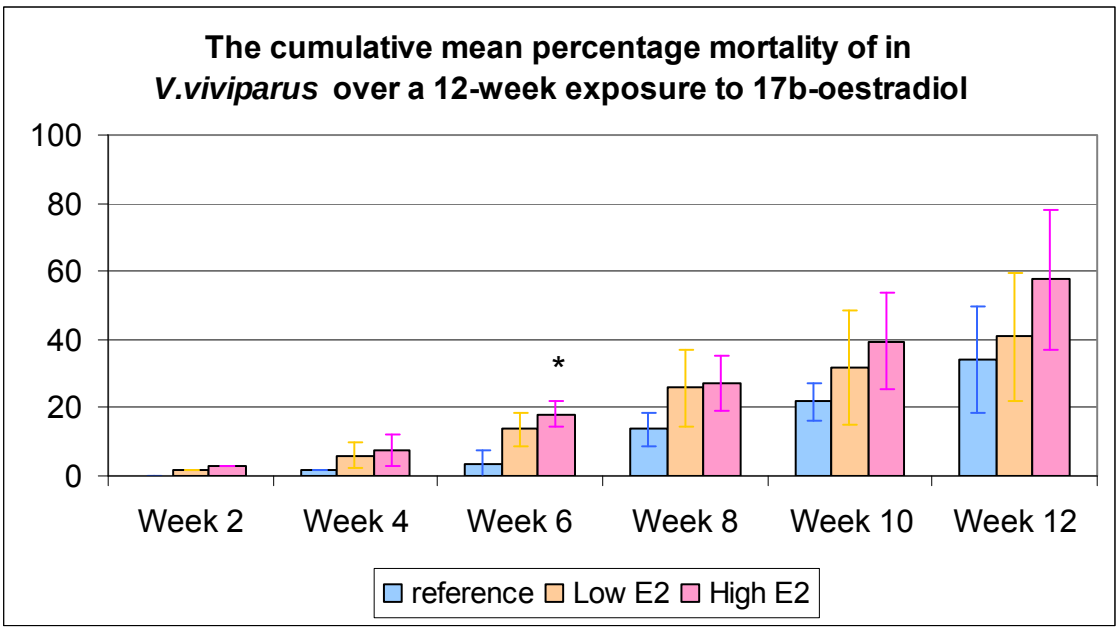

Figure 10. Percentage mortality of *V. viviparus* over a 12-week exposure to E2. Each treatment included 6 enclosures with 11 snails (2 males and 9 females) at the start of the exposure. * indicates a statistically significant difference (P<0.05) compared to RW. Error bars denote standard deviation from the mean.

Growth

Almost all groups of snails showed a mean decrease in shell length (along the longest axis) of up to 1.5%. This was probably due to handling wear on the spire tip during sampling events and so was not considered for further analysis. The total mean body weight of the surviving animals decreased by 4 to 10% across the three tanks, but there were no significant differences between tanks (ANOVA, P>0.05).

Reproduction

There was no apparent pattern in the number of juveniles produced over the course of the exposure. However, productivity was low (and variation high) and this makes meaningful interpretation of the results very difficult (Figure 11). The LE2 treatment was significantly different from the RW tank on only one occasion, week 4 (ANOVA, P>0.05). The HE2 tank was significantly different from the RW tank only at week 12 (Kruskal-Wallis, P<0.05). Overall there were significantly more juveniles released in the HE2 tank than the RW tank (ANOVA, P<0.01). Figure 12 shows the number of embryos dissected out per female ('unhatched embryos' and 'shelled embryos' respectively), along with the total mean number of juveniles counted over the exposure.

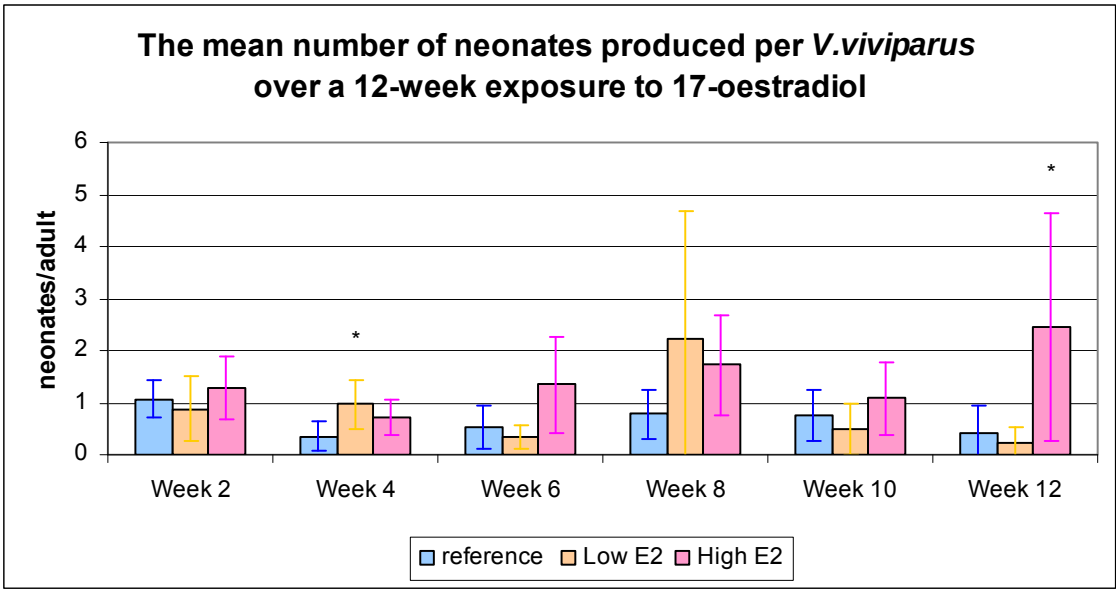

Figure 11. *V. viviparus* juveniles produced per adult over the 12-week E2 exposure. * indicates a statistically significant difference (P<0.05) compared to RW. Error bars denote standard deviation from the mean.

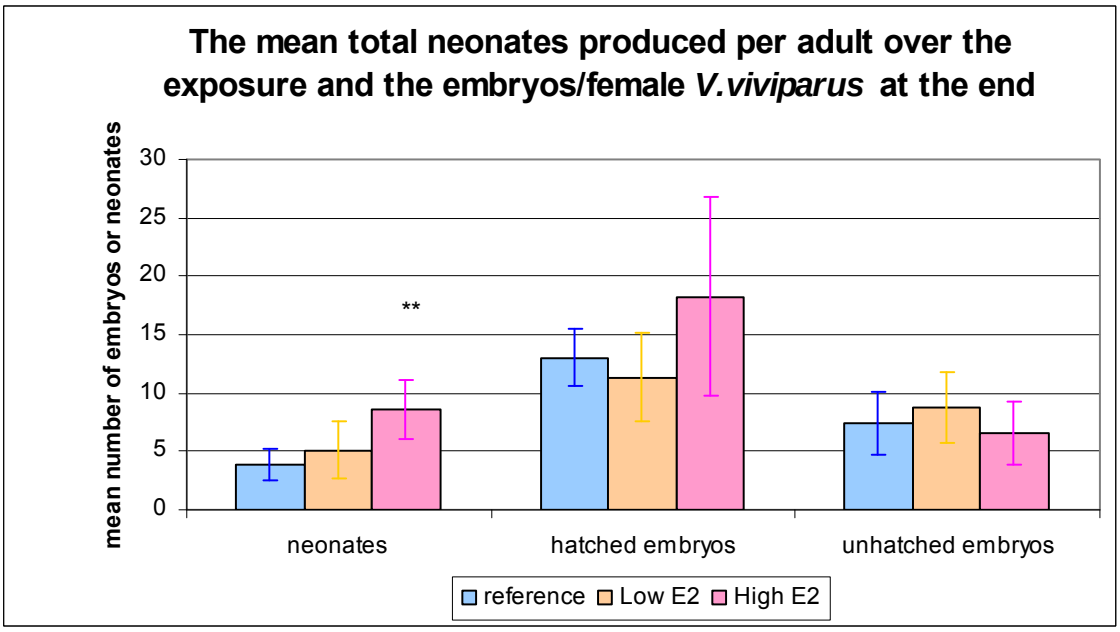

Figure 12. Embryo and neonate production in *V. viviparus* at the end of the 12-week exposure to E2. Unhatched and shelled embryos in the brood pouch were counted, as well as free-living neonates in the exposure tanks. ** indicates a statistically significant difference (P<0.01) compared to RW. Error bars denote standard deviation from the mean.

The number of neonates released is small in comparison with the number of embryos in the reproductive tracts of the females. However, there were no significant differences between tanks in terms of the mean number of unhatched embryos in the females (ANOVA, P>0.05), the mean number of hatched embryos with shells in the females (Kruskal-Wallis, P>0.05), or the mean total number of embryos in the females (ANOVA, P>0.05).

2.1.3 Discussion

In *P.corneus*, the reproductive rate clearly increased in response to the increased temperature and photoperiod experienced by the snails during the summer. The onset of autumn caused a decrease in the snails' reproductive rate. This seasonal decrease in reproductive output was accentuated by exposure to E2, this effect was more marked in the low E2 treatment than the high E2 treatment. Inhibition of egg laying in P.corneus was dose-dependent before the onset of autumn, but in the latter part of the study the underlying seasonal reduction in reproductive rate created the appearance of a 'U-shaped' dose response in the LE2 treatment. *P. corneus* exposed to the high E2 treatment (nominally 100ng/l) showed a significant increase in mortality rate over that of the reference group, indicating exposure to lethal concentrations of a toxicant. An insufficient range of E2 concentrations were tested to establish whether, or not, this was a real non-monotonic dose response. Such responses can occur when there are two underlying end-points (Davies *et al.,* 1990), in this case, a possible reproductive induction in the HE2 group due to impending mortality.

In *V. viviparous*, the reproductive rate was very low (and the variation high) which makes meaningful interpretation of the reproductive output results difficult. In this species, there were high rates of mortality across all treatments, which may be the result of exposure to a combination of stressors (both high temperature and chemical exposure). It was necessary to repeat the study to confirm the apparent increase in neonate production with increasing E2 exposure.

2.2 Mesocosm experiment 1b: A preliminary analysis of the morphology and histology of the F1 generation of *V.viviparus* exposed to E2 during their development

2.2.1 Methods

On completion of the adult reproduction study the F1 generation were exposed to the three treatments for a further week. The tanks were then drained (in September) and a proportion of the F1 snails were removed from each tank. By this time the snails were three months old or younger. All the remaining snails were then depurated for 5 - 6 months in river water by which time they were between 6 and 9 months old, depending on when they hatched. The tanks were drained once more, in the following March, and all the remaining snails were sampled for histology.

2.2.2 Results

Table 3 gives the numbers of snails, from the F1 generation, collected in September and March. In the September samples, the initial results, based on morphology, indicated a dose-dependent increase in the male to female ratio (Table 4), but on analysis there was no statistically significant difference in the proportions of males and females. Chi-squared analysis demonstrated a significant difference in the proportions of male and female viviparids between the low and high dose treatments (P=0.0035). Not all *V. viviparus* F1's were examined histologically to determine their sex, so the results of this analysis were not confirmed histologically. Too few individuals were collected in March to allow valid statistical analysis of the results (Table 5).

Table 3. Number of *V. viviparus* and *P. corneus* F1's collected and fixed in September and March collections.

Removed and fixed	Sep-04		Mar-05	
	V. viviparus	***P. corneus***	***V. viviparus***	***P. corneus***
Stripped river water	8	58	2	57
Low E2 dose	20	60	0	29
High E2 dose	36	37	43	7

Table 4. Number of presumptive male and female *V. viviparus* F1's, from September 2004 collection, deduced from morphological assessment alone.

Sep-04	Total (n)	Male(n)	Female(n)	% Female
Stripped river water	8	0	8	100
Low E2 dose	20	5	15	75
High E2 dose	36	25	11	31

Table 5. Number of presumptive male and female *V. viviparus* F1's, from March 2005 collection, depurated from Oct 2004, deduced from morphological assessment alone.

Mar-05	Total (n)	Male (n)	Female(n)	% Female
Stripped river water	2	1	1	50
Low E2 dose	0	0	0	-
High E2 dose	43	25	18	42

In addition, 23 F1 viviparids (15 from the HE2, 7 from the LE2 and 1 from the RW treatment) were blindly assessed by Dr Burkard Watermann, a snail histology expert, from LimnoMar laboratory in Germany. Of the 15 snails examined from the HE2 treatment, 2 had both male and female gametes present in the gonad. Many snails had poorly differentiated gonads, but this may have been due to the size and age at which they were sampled. Some F1 individuals that were exposed to E2 had both male and female characteristics (e.g. a brood pouch and oocytes are female, vas deferens and spermatogenesis are male); these individuals were deemed to be intersex. The percentage of intersex individuals was 14% in the HE2 tank (4 intersex snails among 29 snails examined) and 5% in the LE2 tank (1 intersex snail among 20 snails examined).

2.2.3 Discussion

Morphological and histological examination of snails exposed to E2 during their development indicated that there are developmental effects on *V. viviparus*. These developmental effects could be classified as 'intersex' (female brood pouch and testicular tissue in gonad). However, the small number of animals collected from the RW treatment (two normal females) means that interpretation of these findings is limited. A greater focus on developmental exposures was made in subsequent mesocosm exposures to E2 and mixtures of EDCs.

2.3 Mesocosm experiment 2a: An exposure of three UK native freshwater gastropod species to E2, in an outdoor mesocosm.

2.3.1 Methods

Experimental set up

In this experiment, both *P. corneus* and *V. viviparus* were exposed to river water or river water dosed with E2 for a period of 16 weeks, from early May until late August 2006. Three mesocosm tanks were used; one tank held river water (control tank, RW), one tank held river water plus the high concentration of E2 (HE2; 100ng/l nominal dose) and another tank held river water plus the low concentration of E2 (LE2; 10ng/l nominal dose). Tap water was used in the dosing reservoir to reduce the level of microbes and algae in the reservoir that might metabolise the solvent and/or dosing chemicals and produce clogging masses, which could affect the dosing rate.

Each tank was fed with water from the River Chelmer. River water was extracted, via a pump, coarsely filtered through gravel and pumped directly to the mesocosm tanks. River water could also be passed through an activated carbon filter to remove any small particles and biologically active materials e.g. estradiol. This method was used previously in an experiment that exposed the same two gastropod species to mixtures of effluent and river water (Clarke, pers. com. 2005). However, as particulate matter and algae are an important food source for the prosobranch snails (*V. viviparus* and *B. tentaculata*) pre-filtration with activated charcoal was not used in this experiment. Water exited the tanks via an overflow pipe and was filtered through activated carbon to remove any EDCs, before being returned to the river. Effluent from the activated carbon filter was monitored for the presence of EDCs, used in the experiment, to confirm their removal.

At the start of the experiment, each tank contained three replicate groups of *P. corneus* (nine individuals per group) and *V. viviparus* (11 individuals per group). Mortality and reproductive output (egg masses and eggs per mass for *P. corneus*, and the number of embryos released by *V. viviparus*) for adult snails, was recorded fortnightly over the 16 week exposure (as described for experiment 1).

Offspring (F1s) produced by *P. corneus* and *V. viviparus* adults were released into the main tanks in which they were conceived. They were later assessed for the effects of ECDs on their development and their reproduction as adults. At the end of the E2 exposure period, all the adult snails were sacrificed, measured and fixed for histopathological analysis. Once the adult snails had been removed, all the F1 snails were collected from each tank and their number and size-classes recorded. A sample of F1s were sacrificed, measured and fixed for histopathological analysis. The remaining F1s were released back into their original tanks; which were now filled with river water only. These F1s were left in the tanks to over-winter and depurate. In the following spring the tanks were scoured for surviving F1s and their numbers recorded. For each treatment three replicate cages were set up, each containing breeding groups of 6 F1 *P. corneus* snails, and three replicate cages, each containing breeding groups of 11 F1 *V. viviparus* snails (2 males and 9 females per group). A breeding study was then conducted over a period of 16 weeks, during which the F1 snails were not exposed to ECDs. F1 reproductive output and mortality were recorded fortnightly. At the end of this F1 breeding study the snails were sacrificed, measured and fixed for

histopathological analysis. Figure 14 shows the experiment timeline and the points at which sampling occurred.

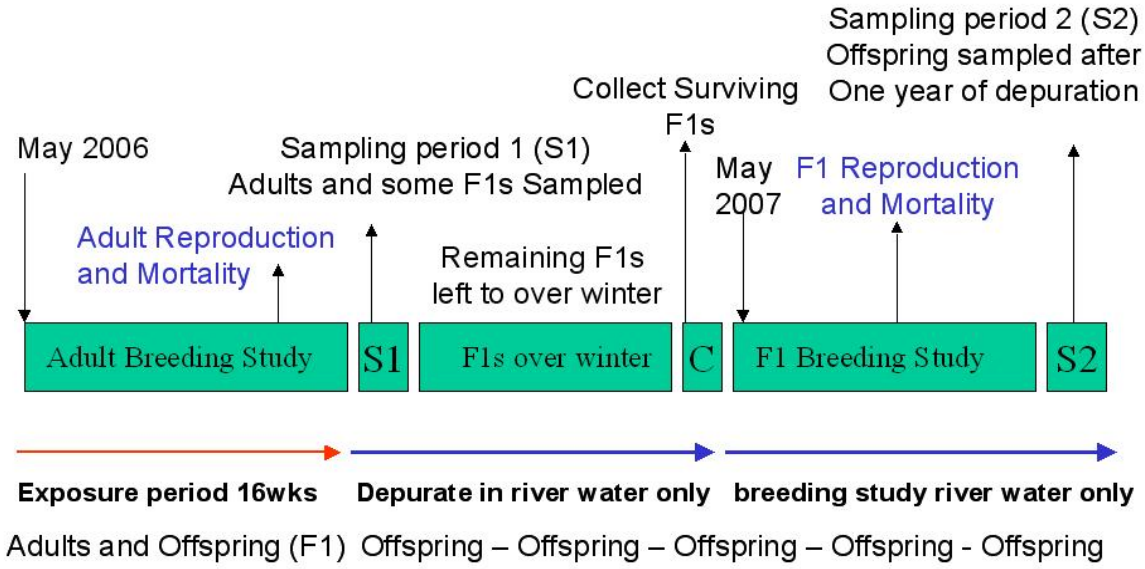

Figure 14. Diagrammatic representation of the timeline for the mesocosm experiments. The period of exposure was 16 weeks, during this time adults and their offspring (F1) were exposed. Adult reproduction and mortality were recorded fortnightly. At the end of the exposure period the first sampling occurred (S1). All adults, and a number of F1s, were sampled and processed to obtain histopathological data on growth and reproductive development/fitness. The remaining F1s were then left to depurate over winter in river water. In spring (2007) the surviving over-wintered F1s were collected and placed in breeding groups for the F1 breeding study. Reproductive output and mortality were recorded fortnightly. At the end of the F1 breeding study, snails were sampled (S2) to collect morphometric and histopathological data. The whole experiment ran from May 2006 until August 2007.

Test organisms

P. corneus were obtained from 'Blades' Biological Supply. They were originally captured from wild stocks in the River Rife, near Worthing. *V. viviparus* were collected from the Grand Union Canal in Cowley, at grid reference TQ055810. In both cases only individuals over 15mm in length were collected as these are considered sexually mature (previous histological analysis had confirmed this).

The feeding regimen was similar to that in the previous mesocosm experiment (experiment 1). The diet for *P.corneus* was altered from fish flake to fish pellets, this was to reduce the flush out rate of the food by the water flow through the mesocosm. The lettuce was exchanged for organically grown carrot. The various weed species that grew in the tanks (blanket weed - *Cladophera spp.*, gutweed - *Enteromorpha spp.* and duckweed *Lemna spp.*) were allowed to grow to a limited extent.

Yeast screen

As well as samples taken for chemical analysis for E2, samples were taken for yeast screen analysis on weeks 2, 6, 8, 10, 12 and 16, from the HE2 and LE2 tanks and the solvent tank (RW+S). The samples (~1000ml) were collected into solvent-rinsed, amber glass bottles. They were transported to Brunel University in cool boxes

containing freezer packs and stored at 4°C, overnight, before extraction onto C18 cartridges. A maximum extraction period of 6 hours was used and the volume extracted during this time was recorded. Standard methods for preparing samples (eluting, drying down and re-suspending) for the yeast screen were followed. Eluted samples were stored in ethanol at 4°C. The yeast screen assays were carried out as described in Routledge and Sumpter, 1996.

2.3.2 Results

Chemical analysis

The measured concentrations of steroids varied between sampling points (Table 7). E2 concentrations in the control tank, at the beginning of the exposure, were approximately the same as those measured at the start of mesocosm experiment 1, despite changing the supply river. However, unlike experiment 1 (where levels gradually increased over time) the E2 levels then fell sharply to concentrations that were more typical of river water.

The measured concentrations of steroids in the mesocosms declined over time. At 8 weeks, only 40% of the nominal value (100ng/l) of E2 was measured in the HE2 tank. By 12 weeks, less than 20% of the nominal concentrations were measured in either of the E2 tanks. At the end of the experiment the measured values in these two tanks were so low as to be effectively indistinguishable from one another.

Table 7. Mean measured concentration of steroid estrogens in the three exposure tanks and the river water pump inlet. All concentrations are measured in nano-grams per litre (ng/l) ± standard deviation. Water chemistry provided by Environment Agency's National Laboratory Service, Nottingham.

	E1	E2	EE2
Low E2 Tank	**4.16 ± 6.07**	**9.32 ± 11.11**	**0.76 ± 0.22**
High E2 Tank	**9.53 ± 11.93**	**60.81 ± 97.00**	**0.79 ± 0.46**
River Water Tank	**1.22 ± 0.46**	**3.34 ± 4.45**	**1.11 ± 0.94**
River Pump inlet	**1.13 ± 0.22**	**0.34 ± 0.07**	**1.70 ± 1.78**

Yeast screen

The results from the yeast screen (E2 EQ) were lower than nominal for the two exposure tanks (Table 8).

Table 8. Measured E2 EQs for RW, LE2, HE2 tanks and the river water inlet (that fed all tanks) using the yeast estrogen screen. Water samples taken at weeks 8, 12 and 16 (highlighted) were split between water chemistry analysis and yeast screen analysis and are directly comparable. No water sample was taken from the river inlet or RW tank on weeks 2 and 4. Mean ± standard deviation. All measured in ng/l. Shared letters (a,b) denote no statistically significant difference between figures.

	Week 2	Week 6	Week 8	Week 10	Week 12	Week 16	Mean
RW	-	-	0	1.67	0	1.75	0.86 ± 0.99 a
LE2	1.74	1.11	5.98	0.88	0	1.15	1.81 ± 2.12 ab
HE2	6.78	0	14.72	3.66	1.71	6.14	5.5 ± 5.20 b
River inlet	-	-	2.01	0	0	0.95	0.74 ± 0.96 a

Effects of E2 exposure on adult *P. corneus* growth, survival and reproduction

Growth

The mean length and weight of the control population was lower than the exposure groups at the start of the experiment (this difference was not statistically significant). After the exposure period there were no significant differences between the treatment groups in terms of surviving adult shell diameter, total weight or soft body weight. The growth rate of the control populations in mesocosm experiments 1 and 2 were very similar, despite the fact that the exposure in experiment 2 was 4 weeks longer. In the control groups in both experiments, the snails increased in length, along their longest axis, by approximately 20% on average, while their mean weight increased by approximately 60% (Figures 4 and 19). As in experiment 1, the mean gain in weight was lower in both groups exposed to E2 than in the control group. Unlike experiment 1, there was an apparent dose-dependency, but the differences between treatments were not statistically significant. There were no significant differences between the weight or length of snails in the groups exposed to E2 and the control population ($P > 0.05$, ANOVA).

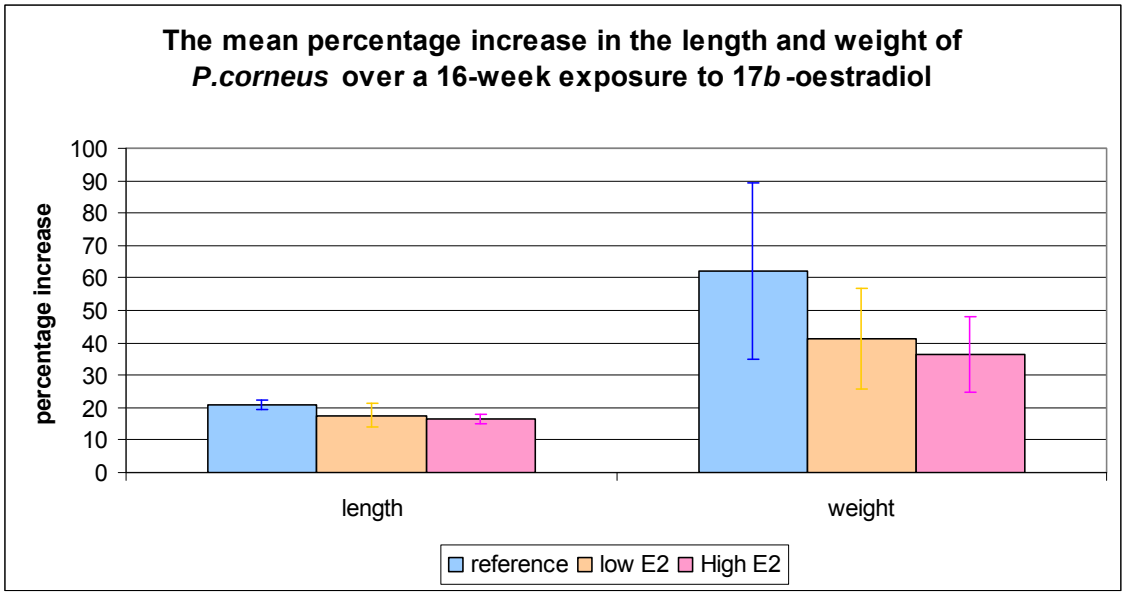

Figure 19. Mean percentage increase in *P. corneus* length, along the longest axis, and weight, during a 16-week exposure to E2.

Mortality

The mortality rate in this experiment was very similar to that observed in mesocosm experiment 1. The mortality rate in the control population was approximately 15% by week 12 (the duration of the first experiment) and slightly exceeded 20% at 16 weeks (see Figure 20). At both time points there was significantly higher mortality in the HE2 group, compared to the RW group. Mortality increased from 40-50% at weeks 10, 12 and 14 (ANOVA, P <0.01 on these occasions) to approximately 70% by week 16 (P <0.001). The high mortality in the HE2 treatment is of interest because the measured E2 concentrations were considerably less than those in mesocosm experiment 1, while the overall exposure period was 4 weeks longer in experiment 2.

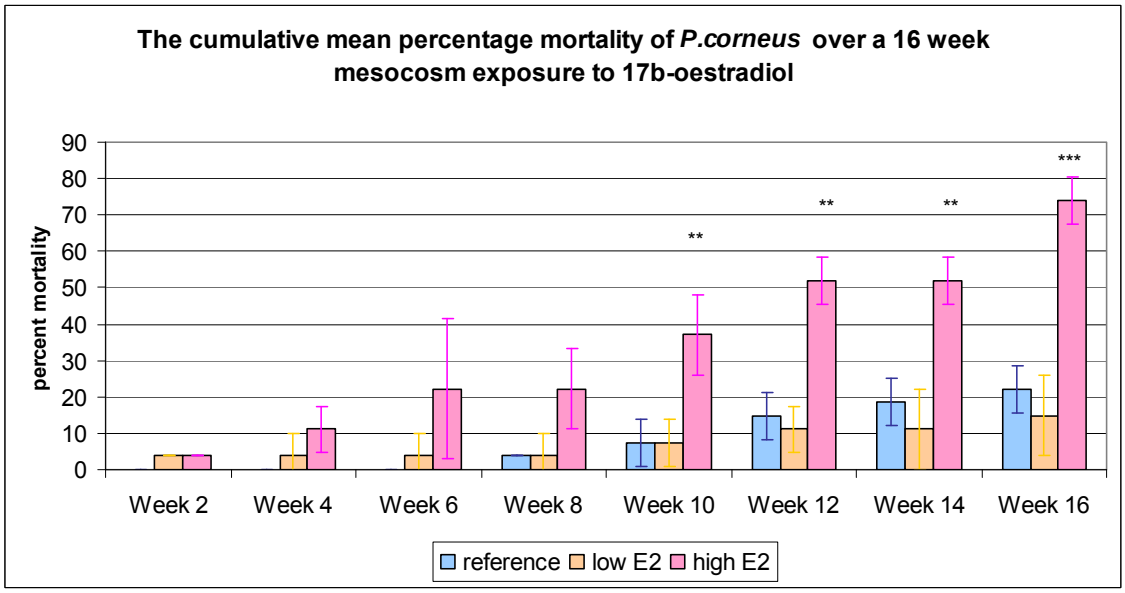

Figure 20. The cumulative percentage mortality of *P. corneus* over a 16-week exposure to E2.

Reproduction

Figure 21 shows the number of eggs produced per surviving adult at each fortnightly sampling point. Adult *P.corneus* showed a complex response to 17β-oestradiol in terms of the numbers of eggs produced. Until week 8 the control population produced the largest number of eggs per adult, reaching a peak of over 500 eggs per individual per fortnight, which ended at week 6 in mid-June. Peaks in egg production also occurred at week 6 in the LE2 and HE2 exposure groups. From week 8 the numbers of eggs laid by the control population gradually declined to less than 50 eggs per snail at week 16. At week 8, the HE2 group produced significantly fewer eggs than the RW group. The two groups exposed to E2 do not decline in the same manner as the RW group, but maintain similar levels of egg production from week 8 until week 14. There were no statistically significant differences between the two groups. At week 12 there was a statistically significant difference in mean egg production between treatments (ANOVA), but a Dunnett's comparison found no significant difference between the mean egg production in the LE2 and RW groups.

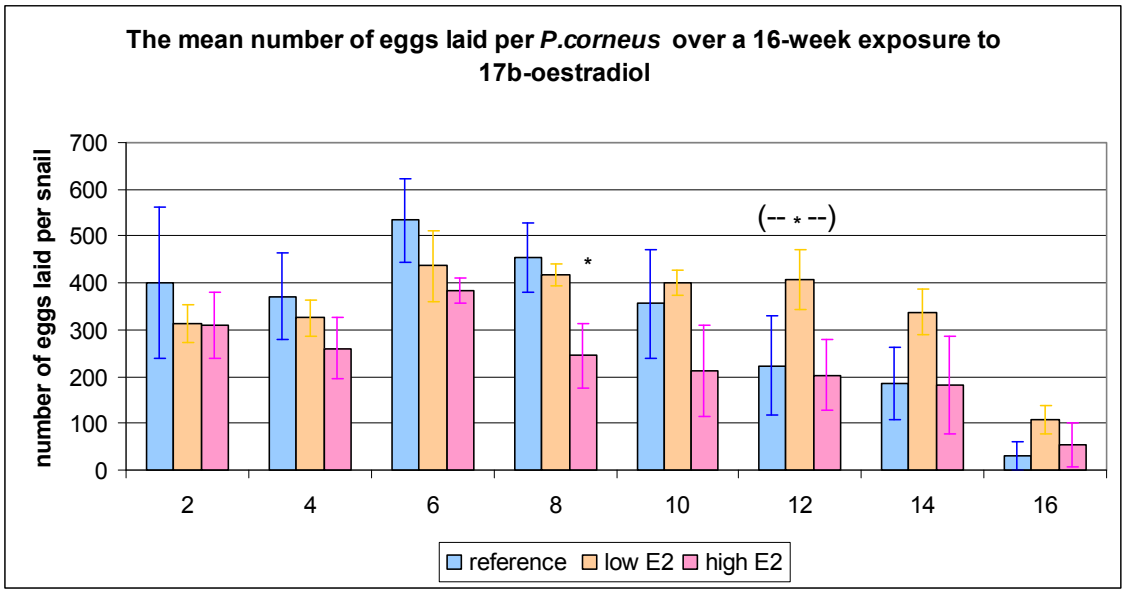

Figure 21. The mean number of eggs produced per fortnight by surviving *P. corneus* during a 16-week exposure to E2. ** denote statistically significance differences (P<0.01) compared to RW. (---*---) indicates that a significant difference was found between the treatments when using an ANOVA test. A statistically significant difference in mean egg production, between LE2 and RW, was not confirmed by a Dunnett's comparison. Error bars are standard deviations from the mean.

Parasitism

No parasites were observed in any of the adult snails from the RW, LE2 or HE2 mesocosms.

Histopathology of adult *P. corneus* exposed to E2

Histological analysis was performed on a sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were examined per adult snail. Due to the complex nature of the *P. corneus* gonad, the analysis was split between different areas, or cell types, within the gonad.

Effects on Acini

All adult *P. corneus* examined (from all treatment groups) had Sertoli cells attached to the acini walls. However, a dose-dependent decrease in normal activity in the vitellogenic area (maturing oocytes with follicle cells and/or sperm with Sertoli cells) was found (Table 10). An analysis of the mean scores, by Oneway ANOVA, found no significant difference between the three treatments (P = 0.060).

Adult snails from the RW and LE2 exposures had the highest and lowest amounts, respectively, of cell cover (developing oocytes, sperm and supporting cells) over the acini walls (Table 11). Analysis of the mean scores, using Oneway ANOVA, found a significant difference between the groups (P = 0.005) and post hoc analysis of LSD found a significant difference between the RW and LE2 snails (P = 0.001), but not between the RW and HE2 (P = 0.111) or between the two E2 treatments (P = 0.115). All adult *P. corneus* examined (from all treatments) had intact acini walls.

Table 10. Percentage of adult *P. corneus* snails with varying levels of active vitellogenic areas after four months exposure to river water only (RW), river water plus a low concentration of 17β-estradiol (LE2) or river water plus a high concentration of 17β-estradiol (HE2). Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail. Shared letters (a, b) denote no statistically significant differences between groups.

Percent of acini with active vitellogenic area	Score	Percentage of adult snails from the river water exposure (RW)	Percentage of adult snails from the low E2 exposure (LE2)	Percentage of adult snails from the high E2 exposure (HE2)
<10%	1	0	0	14.5
10-30%	2	0	20	0
30-50%	3	10	30	14.5
50-70%	4	50	30	71
>70%	5	40	20	0

Table 11. Percentage of adult *P. corneus* snails with varying levels of cell cover over acini walls after four months exposure to RW, LE2 or HE2. Histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail. Shared letters (a, b) denote no statistically significant difference between groups.

Percent of acini wall covered by germ cells and/or supportive cells	Score	Percentage of adult snails from the river water exposure (RW)	Percentage of adult snails from the low E2 exposure (LE2)	Percentage of adult snails from the high E2 exposure (HE2)
<10%	1	0	10	0
10-30%	2	0	20	29
30-50%	3	40	50	14
50-70%	4	10	20	43
>70%	5	50	0	14
		A	B	a,b

Effects on spermatogenesis

The HE2 exposure had the highest proportion of snails where immature cell types (Sertoli cell, spermatogonia, spermatocytes and spermatids) were sloughing in to the lumen of the acini (Table 12).

Sertoli cells sloughing into lumen

Adult snails from the RW treatment had the fewest sections of gonad where Sertoli cells were sloughing into the acini lumen, whereas snails from the HE2 treatment had the highest number (Table 12). Analysis of the mean number of affected sections (Kruskal-Wallis Test) found a significant difference between the groups (P = 0.025). Post hoc analysis, using the Mann-Whitney Test, found a significant difference between the RW and HE2 treated snails (P = 0.010), but not between the RW and LE2 (P = 0.133) or between the LE2 and HE2 treatments (P = 0.105).

Spermatogonium sloughing into lumen

Sloughing of spermatogonia into the lumen was most frequent in snails exposed to HE2 and least frequent in snails exposed to RW (Table 12). The Kruskal-Wallis Test found there was no significant difference between the three treatments (P = 0.095).

Spermatocyte sloughing into the lumen

Snails from the HE2 treatment had the highest number of sections of gonad where spermatocytes were sloughing into the acini lumen. Snails from the LE2 and RW treatments had similar levels of spermatocyte sloughing (Table 12). An ANOVA test found no significant difference between the three groups (P = 0.094).

Spermatids sloughing into the lumen

In all the treatments spermatids were frequently found sloughed into the lumen. However, snails exposed to HE2 were more severely affected than those from the LE2 or RW treatments (Table 12). A Oneway ANOVA found no significant difference, in the mean number of affected sections, between the three treatment groups (P = 0.226).

Table 12. Percentage of adult *P. corneus* snails with varying levels of sloughing of immature spermatogenic cells into the acini lumen. Measurements made after four months exposure to RW, LE2 and HE2. The histological analysis was performed on a sub-sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail. Shared letters (a, b) denote no statistically significant difference between groups. Numbers = P value.

Number of sections affected per	Percentage of adults affected by Sertoli cell sloughing into lumen			Percentage of adults affected by spermatogonium sloughing into lumen			Percentage of adults affected by spermatocyte sloughing into lumen			Percentage of adults affected by spermatid sloughing into lumen		
	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2
None	70	40	14	20	10	0	40	50	0	30	30	14.3
1 out of 4	20	30	29	10	10	0	10	20	29	20	30	0
2 out of 4	10	20	57	10	0	0	30	0	0	0	30	28.6
3 out of 4	0	10	0	10	20	0	10	0	14	30	0	28.6
4 out of 4	0	0	0	50	60	100	10	30	57	20	10	28.6

Spermatozoa only

There was a dose dependent decrease in the number of sections that just contained mature spermatozoa in the acini lumen (Table 13). None of the sections from snails exposed to HE2 had only mature spermatogenic cells in the lumen. Analysis of the mean number of sections with only spermatozoa in the lumen (Kruskal-Wallis Test) found there were no significant difference between the three treatments (P = 0.197).

Table 13. Percentage of adult *P. corneus* snails with different numbers of sections of gonad where only mature spermatozoa were present in the acini lumen, after four months exposure to RW, LE2 or HE2. Histological analysis was performed on a sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail.

No. of sections with only mature spermatozoa in the acini lumen	Percentage of adult snails from the river water exposure (RW)	Percentage of adult snails from the low E2 exposure (LE2)	Percentage of adult snails from the high E2 exposure (HE2)
none	60	70	100
1 out of 4	20	10	0
2 out of 4	10	0	0
3 out of 4	0	10	0
4 out of 4	10	10	0

Overall, the percentage of acini (per section of gonad) where immature spermatogenic cells were sloughing into the lumen, increased in a dose dependent manner. However, Oneway ANOVA of the mean scores found no significant difference between the three groups (P = 0.124).

Effects on oogenesis

The proportions of different stages in oocyte development, that were present in the gonad, were similar across all treatments (Table 15). There were no significant differences, for any of the developmental stages, between the treatments.

Table 15. The mean percentage of different stages in oogenesis, in adult *P. corneus*, after four months exposure to RW, LE2 or HE2. Histological analysis was performed on a sample of adults from each treatment (RW, n = 10; LE2, n = 10; HE2, n = 7). Four sections of gonad were analysed per adult snail. Stage 1 (oogonium) is the earliest stage of oogenesis, stage 5 is ready to be ovulated. The oogenesis stages are based on the description by de Jong-Brink et al. (1979).

Stage of oocyte maturation	Mean percent of oogenesis stage after river water exposure	Mean percent of oogenesis stage after low E2 exposure	Mean percent of oogenesis stage after high E2 exposure
Stage 1	33.5 ± 10.0	31.7 ± 5.7	35.7 ± 8.6
Stage 2	21.7 ± 4.6	20.9 ± 3.2	20.6 ± 1.6
Stage 3	20.8 ± 5.5	21.1 ± 2.3	23.0 ± 7.3
Stage 4	11.0 ± 4.7	12.4 ± 5.5	9.0 ± 5.4
Stage 5	0.8 ± 1.2	0.3 ± 0.4	0.9 ± 0.8
Degenerating	12.1 ± 5.3	13.5 ± 6.5	10.8 ± 3.6

Effects of E2 exposure on adult *V. viviparus* growth, reproduction and survival

Survival

The mortality rates for *V. viviparus* in the second mesocosm experiment (approximately 30% in the reference population and 60% in the HE2 group) are very similar to those seen in mesocosm experiment 1 (Figures 10 and 24), except that there was no dose-

dependent response on this occasion. Between week 14 and week 16 the mortality in the HE2 group doubled, which included 6 snails being found dead in one replicate.

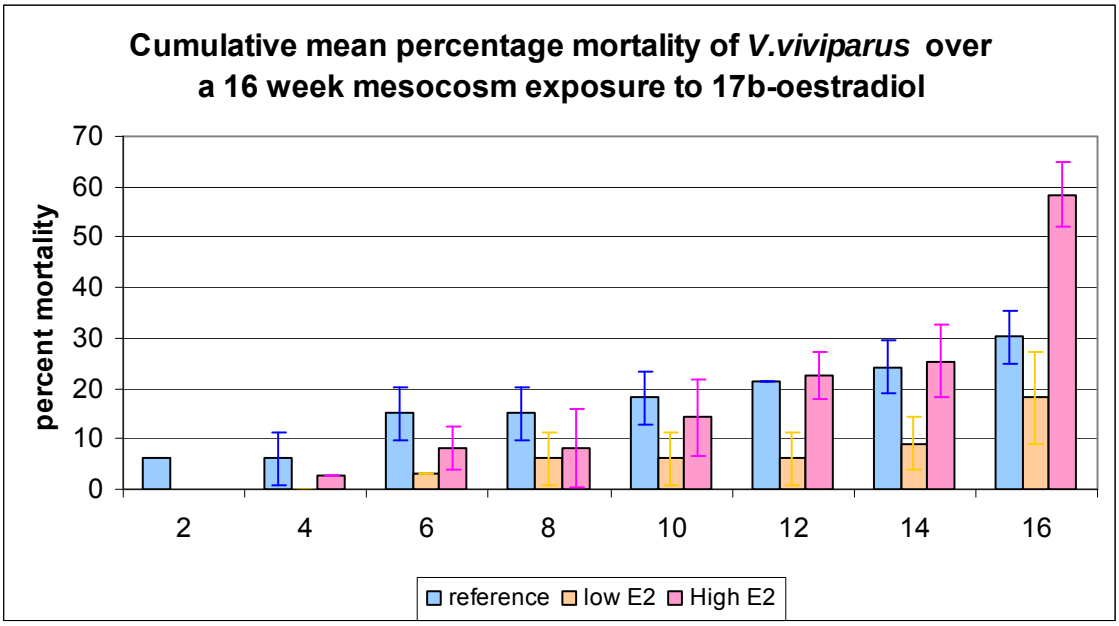

Figure 24. The cumulative percentage mortality of *V. viviparus* during a 16-week exposure to E2.

At the start of the experiment there were no significant differences between the control and exposure groups in terms of their length, along the longest axis, or their weight (Kruskal-Wallis for length, ANOVA for weight, P >0.05). As in experiment 1, the snails showed no increase in the length of their longest axis, but appeared to shrink due to the loss of the spire, probably during handling. All the groups lost weight and the proportion of weight loss (3 to 9%) was similar to that seen in experiment 1 (4 to 10%).

Reproduction

The numbers of neonates produced by *V. viviparus* were similar to those reported in experiment 1 (approximately one neonate per adult female per week) with the exception of the first sampling point (week 2) when almost three times this number were produced by all the groups (Figure 22). It is clear that E2 had no effect on the number of neonates produced, except at week 14, when significantly more juveniles were produced by the HE2 group (P <0.05, ANOVA).

The cumulative number of neonates released per snail was highest in the snails exposed to HE2. Snails exposed to RW released 16.6 neonates each, while snails exposed to LE2 and HE2 released 16.2 and 19.2, respectively. The numbers of neonates released per snail were similar across the three treatments until week 12 (RW = 15.4; LE2 = 15.3; HE2 = 15.9). After this snails exposed to HE2 released a larger number of neonates. This effect is illustrated in figure 23, which shows the mean number of neonates produced per adult in the second half of the exposure.

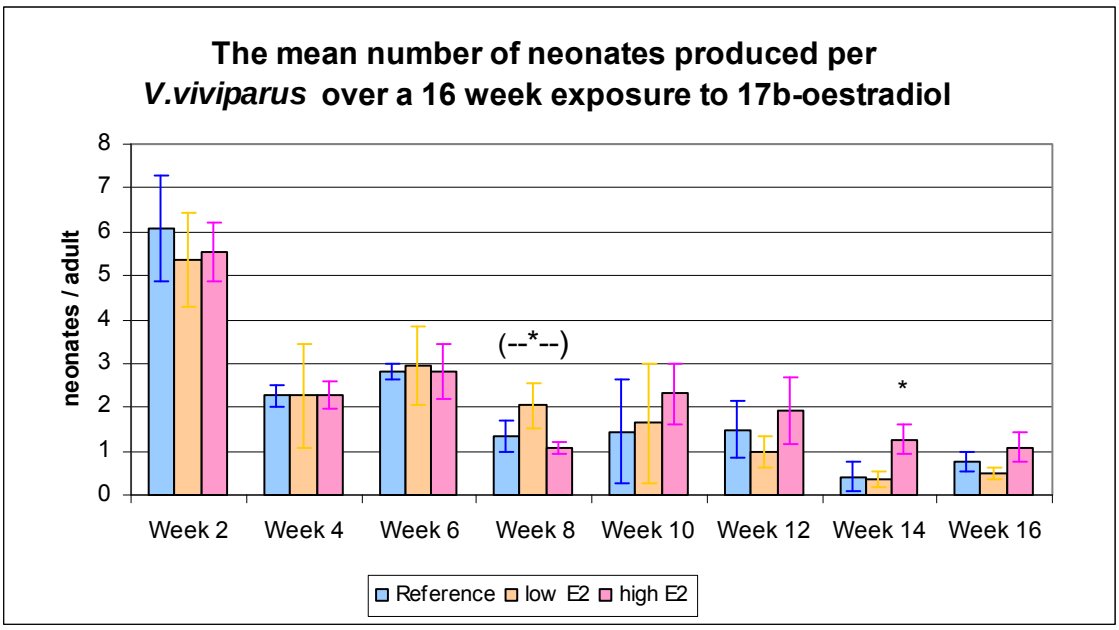

Figure 22. Adult *V. viviparus* exposed to RW, LE2 or HE2. Mean number of neonates released per adult snail during a 16 week exposure period. Error bars give standard deviation. * indicates a statistically significant difference from other treatments (P>0.05 LSD).

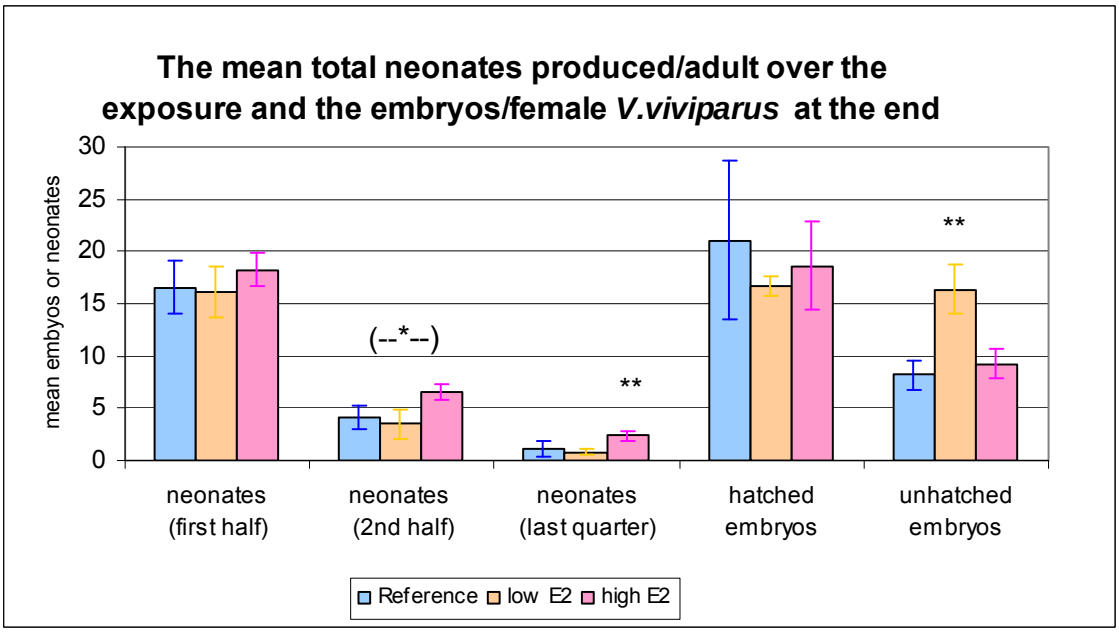

Figure 23. The number of neonates produced, by surviving adult *V. viviparus*, during a 16 week exposure to E2, and the number of embryos found in the brood pouches of females at the end of the exposure * = P <0.05 (ANOVA) ** = P <0.01 (ANOVA). Error bars denote standard deviation from the mean.

When females were dissected at the end of the exposure, similar numbers of hatched embryos were found within the brood pouch (approximately 15 per female) in all groups (Figure 23). The numbers of unhatched embryos found in the brood pouches of control females (approximately 8 per female) were similar to those recorded in experiment 1. However, there were significantly more unhatched embryos per female in the LE2 group than in the control or HE2 groups (P <0.01, ANOVA).

Effects of E2 exposure on adult *V. viviparus* gonad histopathology

All adult males (RW, n= 2; LE2, n= 5; HE2, n= 1) and a sample of 10-11 females per treatment (RW, n= 11; LE2, n= 11; HE2, n=10) were examined histologically.

Effects on oogenesis

The mean number of oocytes, per section of gonad, increased in a dose-dependent manner (Table 18). However, there were no significant differences between the three treatments (P = 0.576, ANOVA).

Table 18. Mean number of oocytes (±SD) counted per section of gonad in *V. viviparus* adult females following exposure to RW, LE2 or HE2. Ten sections were analysed per adult female. RW n= 11, LE2 n= 11, HE2 n=10.

	River water (RW)	Low E2 (LE2)	High E2 (HE2)
Mean oocytes per section analysed	7.5 ± 5.8	9.0 ± 4.5	10.6 ± 9.2

Effects on spermatogenesis

The mean spermatogenesis score was lower in snails exposed to E2, compared to RW males (Table 19). However, too few animals were examined to draw any firm conclusions (RW, n=2; LE2, n=5; HE2, n=1) or to allow valid statistical analysis.

Table 19. Mean (± standard deviation) spermatogenesis score per section of gonad in *V. viviparus* males exposed to River Water only, LE2 or HE2. Ten sections were examined per adult male. RW n= 2, LE2 n= 5, HE2 n= 1.

	River Water (RW)	Low E2 (LE2)	High E2 (HE2)
Mean spermatogenesis score per section analysed	10.0 ± 0.0	9.78 ± 0.2	9.9

Parasitism

The majority of adult *V. viviparus* had some degree of parasite infection. These were either encysted parasites (metacercaria) or infections in the digestive tissue (sporocyst stage). Only three of the adult *V. viviparus* females that were examined had no parasitic infection (RW = 1; HE2 = 2). Encysted parasites (metacercaria) in the muscular tissue of the head and the foot were most frequent, but infections in the digestive tissue were also found.

In adult female *V. viviparus*, the intensity of infection by encysted parasites (number of parasites or area infected) was highest in the RW group and lowest in the HE2 group. In the one HE2 female found with digestive tissue infection, the infection covered more than 70% of the tissue. The infections seen in the RW females were less severe with between 30 and 50% of the tissue affected.

Only two adult *V. viviparus* males had no parasitic infection (both from the LE2 treatment). None of the males had an infection of the digestive tissue, the majority had encysted parasites. The intensity of infection by encysted parasites was highest in the RW treatment. Snails from LE2 had a lower level of infection. There was only one infected male from the HE2 treatment. Figure 25 presents this data as a histogram,

although the number of animals is too few to draw any firm conclusions (RW, n=2; LE2, n=5; HE2, n=1).

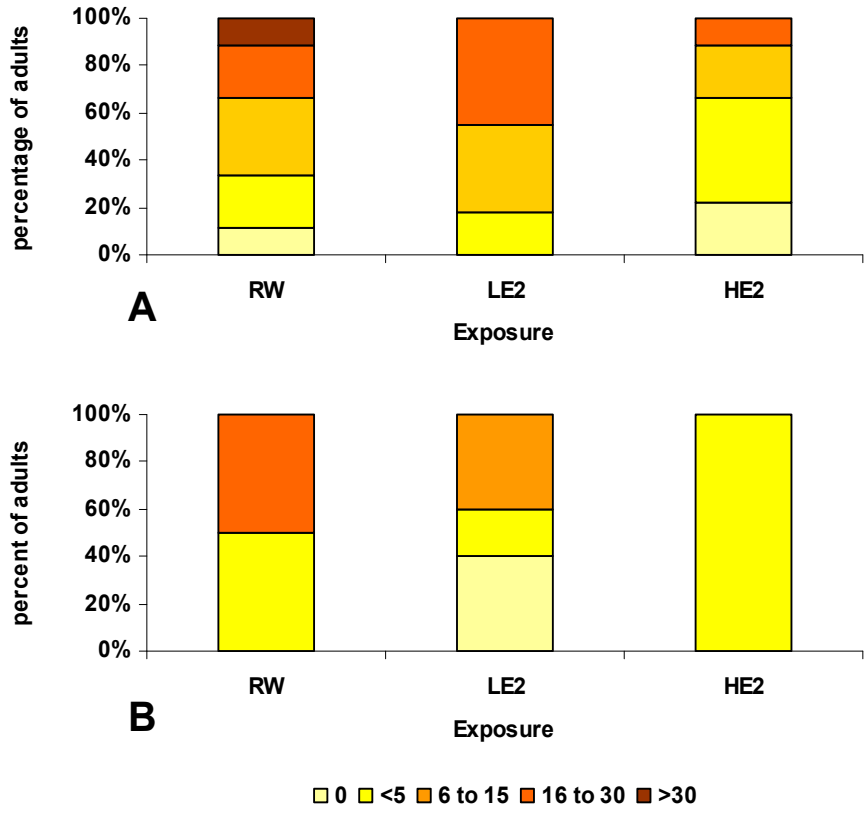

Figure 25. Histograms of the intensity of encysted parasite infections in adult *V. viviparus* after four months exposure to RW, LE2, or HE2. (A) Histogram of the percentage of females with varying intensities of parasite infection. (B) Histogram of the percentage of males with varying intensities of parasite infection. Ten sections per adult snail were analysed. Pale yellow indicates no parasites found, yellow indicates less than 5 parasites per section, light orange indicates 5-15 parasites per section, dark orange indicates 15-30 parasites per section, brown indicates more than 30 parasites per section.

2.3.3 Discussion

These results show that the actual concentrations of dosed oestrogen, in the treatment tanks, were highly variable throughout the study and tended to decrease over time. EE2 (which was not dosed) was also intermittently present in the inlet. The variation in the actual concentration of dosed oestrogen was not surprising given the high density of macro algal growth in the tanks when compared to experiment 1. In the second half of the exposure period (weeks 8 to 16) the rate of weed growth was quite exceptional and necessitated the manual removal of large quantities of plant and algal material. There was also microbial growth in the dosing reservoirs, despite the use of tap water as diluent. To examine the effect of this more closely, the yeast oestrogen screen was used to measure the E2 equivalents in the dosing reservoirs every 3-4 days during July. Only 30 minutes after mixing the oestrogen stock with the tap water, the oestrogenic activity in the dosing reservoir had changed. After 72 hours, the activity of the high dose had reduced to around 50% of what was expected. Despite this dramatic

fall in activity, the biological results do suggest that the snails in the high dose tank were receiving a higher dose than those in the low dose tank, as the mortality rates were consistent with those previously seen for estradiol treatments.

In adult *P.corneus* there were no significant reductions in egg production until week 8, when the higher exposure group laid significantly less eggs than the control group (ANOVA, $P<0.01$). Up to week 12 the snails exposed to HE2 consistently laid fewer eggs than the other two treatment groups. However, at week 12 snails in both the RW and the HE2 treatments laid significantly fewer eggs per snail than the snails exposed to LE2. This trend continued up to the end of the exposure. There were no significant differences, in the cumulative number of eggs laid per snail, between the three treatment groups ($P = 0.066$). The responses to E2 appeared to differ depending on the time of the year they were measured (temperature and photoperiod). The stimulation of egg production in the LE2 treatment was especially evident during the seasonal decline (after the summer solstice peak). A similar response was observed when *P. corneus* were exposed to graded concentrations of treated sewage effluent (Clarke et al. 2009).

In *V. viviparus*, the numbers of juveniles produced per adult were similar across treatments until week 12, when offspring production increased in the HE2 treatment, relative to the control tank. This parallels the pattern of egg production in *P. corneus.* A similar pattern was seen in mesocosm experiment 1, where the higher exposure group also produced significantly more neonates in the last week of the exposure. This appears to confirm that exposure to E2 stimulates *V. viviparus* to produce more juveniles than expected in the late summer and autumn.

The reasons for the seasonal differences in egg and embryo production, when these snail species are exposed to E2, are not known. It is probable that the snails are responding to both chemical and physical stressors (e.g. temperature changes) by adopting a form of semelparity; where a high rate of reproductive output precedes mortality. This is an adaption to ensure survival of the population in the presence of an environmental stressor that might cause adult death. However, “reproduction in the face of adversity” normally occurs at the expense of body growth and the snail’s energy reserves, resulting in death. The adult *V. viviparus* did appear to lose weight during the experiment, but exposure to E2 did not exacerbate this situation. Alternatively, it may be that the snails that died during the experiment were also the biggest snails. In contrast, the *P. corneus* snails appeared to gain weight during the exposure. Unless the snails that died were also the smallest snails. Taken together, the results suggest that more complex reasons than just toxicity account for the differences in reproductive rates between the exposed and non-exposed snails.

It is possible that E2 affects the snails’ ability to reproduce through its direct action on the neuroendocrine system, as seen with natural and synthetic steroid hormones and alkylphenolic estrogenic mimics in fish and other vertebrates. Stimulatory and inhibitory effects of some of these chemicals, on snail reproduction, have been reported. For example, Oehlmann et al. (2000) reported stimulatory effects of the synthetic steroid oestrogen (EE2), the alkylphenolic chemical octylphenol (4-tert-OP) and bisphenol-A, on reproduction in the tropical snail *Marisa cornuarietis* and the marine prosobranch *Nucella lapillus* (EE2 not tested). In the case of BPA there was evidence that these effects are dependent on the season *(*Oehlmann et al. 2006*).* Duft et al. (2003) reported the stimulatory effects of 4-tert-OP, nonylphenol (NP) and BPA on the production of new embryos in the brood pouch of the freshwater mud snail *Potamopyrgus antipodarum*.

Little is known about the role of steroid estrogens in the endocrine control of snail reproduction and how these chemicals might act to disturb reproduction. The

hypothesis, that chemically induced disruption of the endocrine system occurred in the snails exposed to E2, is supported by the preliminary evidence of a dose-dependent inhibition of maturation and histopathological abnormalities in the adult gonad of *P. corneus*. (This effect was not seen in *V. Viviparus*). The Sertoli cells, which support spermatogenesis, seem to be especially sensitive. In adult snails exposed to HE2 there was a significant increase in the number of Sertoli cells and spermatogonia that were found sloughed into the acini lumen, where they are presumed to be no longer functional. Negative effects of E2 exposure on Sertoli cells have been reported in several vertebrates. Rasmussen et al. (2005) reported that E2 exposure (0.5μg/l E2, continuous flow through) disrupted Sertoli cell structure and function in the eelpout (*Zoarces viviparus*), which resulted in impaired spermatogenesis and the lobular structure of the testis. Proliferation of, and degenerative changes to, Sertoli cells were observed in adult fathead minnows (*Pimphales promelas*) exposed to E2 (0.0625-1000nM) for 14 days (Miles-Richardson et al. 1999). Exposure of adult male rats to E2 (0.1mg/kg) for 10 days significantly reduced their sperm count and the number of litters they sired (Aleem et al. 2006). There were also indications of Sertoli cell dysfunction (immature spermatogenic cells found in the lumen) in *P. corneus* that were exposed, either as adults or during development, to treated sewage effluents (Clarke et al. 2009).

2.4 *Mesocosm experiment 2b:* An analysis of the morphology and histology of the F1 generation developmentally exposed to E2

2.4.1 Methods

At the end of the adult exposure experiment (September 2006), all of the F1 snails were collected and measured. Fifty *V. viviparus* and 40 *P. corneus* snails, that were over 10mm in length, were sacrificed for histological analysis. The mesocosm tanks were re-filled with river water and the remainder of the F1 snails were allowed to depurate over the winter. In the following March the surviving F1 snails were collected and measured. In April, for each species, three replicate mesh sleeves were established for the control and two E2 exposure groups. The sleeves contained either six *P.corneus* or 11 *V.viviparus* (9 females and 2 males). Only snails with shells over 10mm in length (sexually mature) were used in the F1 breeding study (where possible). Reproductive output and mortality were measured fortnightly using the same protocol as used for the adult snails. The F1 *V. viviparus* breeding study continued for 19 weeks. At the end of this period, all the surviving F1 snails were sacrificed for histological analysis.

2.4.2 Results

Effects of E2 exposure on F1 *P.corneus* survival, growth and development.

Survival and growth

There was a dose-dependent increase in the total number of F1 *P. corneus* in the mesocosm tanks at the end of the experiment, RW (164), LE2 (274) and HE2 (483). The F1s from the HE2 treatment were significantly smaller (shell diameter and aperture) and lighter (total weight and soft body weight) than those from the LE2 and RW treatments (Figure 27).

The percentage of snails with shells over 10mm in diameter (normal minimum size for sexually active snails) was highest in the RW tank (73%), while in both the HE2 and LE2 tanks, 46% of the snails had shells over 10mm in diameter (Figure 27).

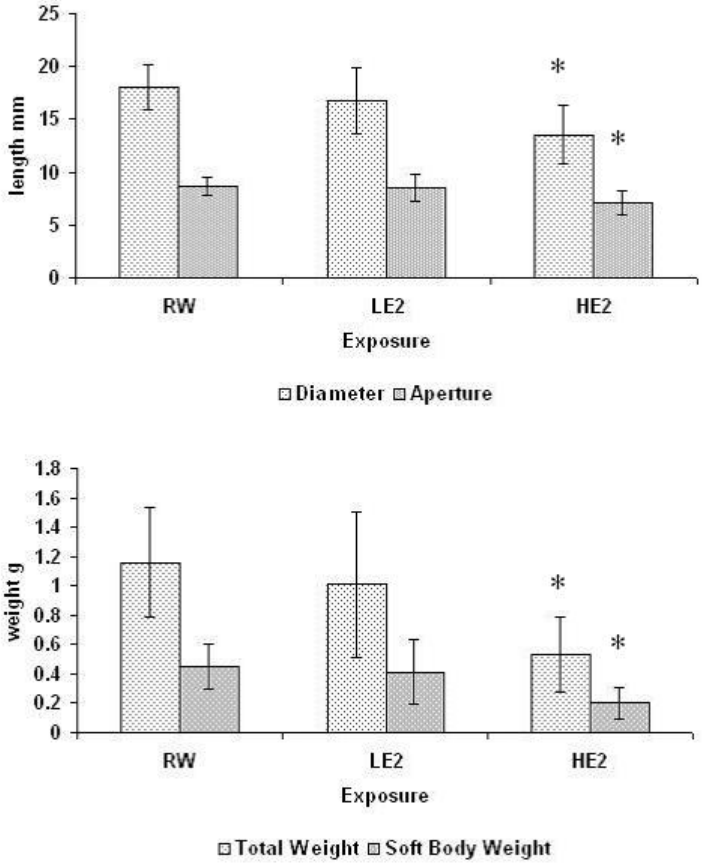

Figure 27. (A) Mean shell length and aperture and (B) mean total weight and soft body weight of F1 *P. corneus* sampled from the three treatment tanks in September 2006. Error bars indicate standard deviation around the mean. * indicates a statistically significant difference (P<0.05) from other treatments.

Histopathology of *P. corneus* F1 gonads immediately after exposure to E2.

Due to the complex nature of the *P. corneus* gonad, the analysis was split between different areas, or cell types, within the gonad. All stages of oogenesis and spermatogenesis were found in the majority of snails from all treatments. However, exposure to E2 did increase disruption to gonad structure and function.

Effects on Acini

One snail from the HE2 treatment had no Sertoli cells attached to the acini walls in four out of five of the gonad sections examined. All other snails (from all three treatments) had Sertoli cells attached to the acini walls in five out of five gonad sections. There was a dose-dependent decrease in normal activity in the vitellogenic area (maturing oocytes with follicle cells and/or sperm with Sertoli cells) (Table 24). A Mann-Whitney Test on the mean scores identified significant differences between the RW and E2 exposed snails (LE2, P = 0.008 and HE2, P <0.001) and between the LE2 and HE2 exposed snails (P = 0.002).

Table 24. Percentage of F1 *P. corneus* snails with different levels of activity in the acini vitellogenic area, after exposure to RW, LE2 or HE2 during their development. Histological analysis was performed on a sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Letters (a, b, c) denote statistically significant differences between groups.

Percent of acini with active vitellogenic area	Score	Percentage of F1 snails from the river water exposure (RW)	Percentage of F1 snails from the low E2 exposure (LE2)	Percentage of F1 snails from the high E2 exposure (HE2)
<10%	1	0	0	16
10-30%	2	0	0	11
30-50%	3	0	20	47
50-70%	4	21	35	21
>70%	5	79	45	5
		a	b	C

Snails exposed to HE2 had more frequent disruption to the acini wall than snails exposed to LE2 or RW. Snails exposed to LE2 had the least disruption (Table 25). A Kruskal-Wallis test of the number of affected sections found a significant difference between treatments (P = 0.009). Post hoc analysis, using a Mann-Whitney test, found a significant difference between the LE2 and HE2 treatments (P = 0.003) but no significant difference between the HE2 and RW (P = 0.088) or LE2 and RW treatments (P = 0.166).

Table 25. Percentage of F1 *P. corneus* snails with different levels of disruption to the acini wall, after exposure to RW, LE2 or HE2 during their development. Histological analysis was performed on a sub-sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Shared letters (a, b) denote there was no statistically significant difference between these groups.

Number of sections affected by acini wall disruption	Percentage of F1 snails from the river water exposure (RW)	Percentage of F1 snails from the low E2 exposure (LE2)	Percentage of F1 snails from the high E2 exposure (HE2)
None	73	90	47
1 out of 5	11	5	19
2 out of 5	0	5	0
3 out of 5	3	0	10
4 out of 5	11	0	14
5 out of 5	0	0	10
	a	B	a, b

Snails from the HE2 treatment had the least cell coverage (developing oocytes, sperm and supporting cells) on the acini walls. Snails from the RW treatment had less than those from the LE2 treatment (Table 26). A Oneway ANOVA test of the scores identified a significant difference between groups (P <0.001). A post hoc test of LSD found that the score in LE2 snails was significantly higher than the HE2 or RW snails

(P <0.001 and <0.001), but there was no significant difference between the HE2 and RW exposed snails (P = 0.227).

Table 26. Percentage of F1 *P. corneus* snails with different degrees of cell coverage on the acini wall, after exposure to RW, LE2 or HE2 during their development. Histological analysis was performed on a sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Shared letters (a, b) denote there was no statistically significant difference between those groups.

Percent of acini wall covered by germ cells and/or supportive cells	Score	Percentage of F1 snails from the river water exposure (RW)	Percentage of F1 snails from the low E2 exposure (LE2)	Percentage of F1 snails from the high E2 exposure (HE2)
<10%	1	0	0	20
10-30%	2	47	5	35
30-50%	3	41	21	35
50-70%	4	12	53	10
>70%	5	0	21	0
		a	b	a

Effects on spermatogenesis

Sertoli cells sloughing into lumen

The number of sections where Sertoli cells were sloughed into the lumen was higher in snails from the E2 treatments, compared to those from RW (Table 27). However, there was no significant difference between treatments (P = 0.258, Kruskal-Wallis test).

Spermatogonia sloughing into lumen

Sloughing of spermatogonia into the acini lumen was very common in snails from all three mesocosm tanks. A dose-dependent increase in the percentage of snails with the highest levels of sloughing (five out of five sections affected) was observed (Table 27). A Oneway ANOVA found no significant difference between treatments (P = 0.307).

Spermatocytes sloughing into the lumen

Sloughing of spermatocytes into the lumen was less frequent than sloughing of spermatogonia. The number of sections affected by spermatocyte sloughing was similar in the HE2 and RW treatments, but there was a higher frequency of affected sections in the LE2 treated snails (Table 27). A Oneway ANOVA found no significant difference between treatments (P = 0.119).

Spermatids sloughing into the lumen

There was a dose-dependent increase in the sloughing of spermatids into the lumen (Table 27). There was a significant difference between treatments when tested using a Oneway ANOVA (P = 0.009). Post hoc analysis, using LSD, found snails from the RW treatment to have significantly less spermatid sloughing than snails exposed to HE2 (P = 0.002). There was no significant difference between RW and LE2 (P = 0.082) or between the two E2 doses (P = 0.153).

Table 27. Percentage of F1 *P. corneus* snails with different levels of sloughing of immature spermatogenic cells into the acini lumen, after exposure to RW, LE2 or HE2 during their development. Histological analysis was performed on a sub-sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Shared letters (a, b) denote no statistically significant difference between these groups.

Number of sections affected	Percentage of F1s affected by Sertoli cells sloughing into lumen			Percentage of F1s affected by spermatogonia sloughing into lumen			Percentage of F1s affected by spermatocyte sloughing into lumen			Percentage of F1s affected by spermatids sloughing into lumen		
	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2
None	78.9	55	66.7	5.3	10	4.8	63.2	40	71.4	42.1	20	14.3
1 out of 5	15.8	25	9.5	5.3	0	0	21.1	25	14.35	21.1	25	14.3
2 out of 5	5.3	10	0	10.5	0	0	10.5	25	9.5	21.1	15	14.3
3 out of 5	0	5	14.3	10.5	15	0	5.3	0	4.8	10.5	10	23.8
4 out of 5	0	5	4.8	15.8	15	23.8	0	5	0	5.3	30	4.8
5 out of 5	0	0	4.8	52.6	60	71.4	0	5	0	0	0	28.6
	a	a	a	a	a	A	a	a	a	a	ab	b

Spermatozoa

Over half the snails from all treatments had immature spermatogenic cells in the lumen. Snails from the HE2 treatment had the highest percentage of sections, per snail, where early spermatogenic stages were sloughing into the acini lumen (Table 28). A Kruskal-Wallis test found a significant difference between treatments (P = 0.016). Post hoc analysis, using the Mann-Whitney test, found HE2 treated snails had significantly fewer sections of gonad with only mature spermatozoa in the lumen, compared to snails exposed to LE2 (P = 0.017) or RW (P = 0.005). There was no significant difference between snails exposed to RW and LE2 (P = 0.608).

Table 28. Percentage of F1 *P. corneus* snails with different numbers of sections of gonad containing only mature spermatozoa in the acini lumen, after exposure to RW, LE2 or HE2 during their development. Histological analysis was performed on a sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Shared letters (a, b) denote no statistically significant difference between these groups.

No. of sections with just mature spermatozoa in the lumen	Percentage of F1 snails from the river water exposure (RW)	Percentage of F1 snails from the low E2 exposure (LE2)	Percentage of F1 snails from the high E2 exposure (HE2)
none	52.6	60	90.5
1 out of 5	15.8	15	9.5
2 out of 5	10.5	15	0
3 out of 5	10.5	0	0
4 out of 5	5.3	0	0
5 out of 5	5.3	10	0
	a	a	B

Overall, there was a dose-dependent increase in the number of acini affected by sloughing of immature spermatogenic cells into the lumen (Table 29). Analysis of the scores, using a Kruskal-Wallis test, found a significant difference between treatments (P = 0.003). Post hoc analysis, using the Mann-Whitney Test, found no significant difference between the two E2 treatments (P = 0.232). Snails exposed to RW had significantly fewer acini with immature spermatogenic cells in the lumen, than snails in the LE2 (P = 0.011) and HE2 treatments (P = 0.002).

Effects on oogenesis

In *P. corneus* there was a dose-dependent increase in the ratio of young to old oocytes in snails exposed to E2 during development. These snails had a higher percentage of young oocytes, compared to snails from the RW treatment (Table 30). A Kruskal-Wallis Test found significant differences between the treatments for numbers of stage 1 oocytes (P <0.001). Post hoc analysis, using the Mann-Whitney Test, found that snails from the RW treatment had significantly fewer stage 1 oocytes, compared to snails exposed to E2 (LE2, P = 0.003; HE2, P <0.001), but there was no significant difference between E2 treatments (P = 0.109). A Oneway ANOVA test found significant differences, between groups, in the numbers of stage 2 (P =0.001), 4 (P = 0.032) and degenerating oocytes (P = 0.011). Post hoc analysis, using LSD, found significantly fewer stage 2 oocytes in RW, compared to LE2 (P = 0.001) and HE2 (P = 0.002). The E2 treatments were not significantly different from one another (P = 0.760). There were significantly more stage 4 oocytes in RW snails than HE2 exposed snails (P = 0.010), but there were no significant differences between LE2 and RW (P = 0.088), or LE2 and HE2 (P = 0.357) treatments. There were significantly more degenerating oocytes in RW compared to LE2 (P = 0.027) and HE2 (P = 0.004), but there was no significant difference between the two E2 treatments (P= 0.455).

Table 30. Mean percentage of different stages of oogenesis in F1 *P. corneus*, after exposure to RW, LE2 or HE2 during their development. Histological analysis was performed on a sample of F1s from each treatment (RW, n = 18; LE2, n = 20; HE2, n = 21). Five sections of gonad were analysed per snail. Stage 1 (oogonia) are the youngest stage of oogenesis, stage 5 are ready to be ovulated. The oogenesis stages are based on descriptions by de Jong-Brink et al. (1979). Shared letters (a, b) denote no statistically significant difference between these groups.

Stage of oocyte maturation	Mean percent of oogenesis stage after river water exposure	Mean percent of oogenesis stage after low E2 exposure	Mean percent of oogenesis stage after high E2 exposure
Stage 1	8.3 ± 3.8 a	13.2 ± 4.1 b	18.3 ± 10.5 b
Stage 2	13.7 ± 5.1 a	20.4 ± 7.5 b	19.9 ± 5.0 b
Stage 3	22.3 ± 4.3 a	26.4 ± 7.6 a	23.4 ± 5.9 a
Stage 4	27.7 ± 7.5 a	23.4 ± 7.1 ab	21.2 ± 8.3 b
Stage 5	16.8 ± 9.5 a	10.2 ± 8.0 a	12.5 ± 9.6 a
Degenerating	11.2 ± 7.6 a	6.3 ± 5.3 b	4.7 ± 7.3 b

Effects of E2 exposure on FI *P.corneus* survival, growth, development and reproduction following depuration.

Survival and growth

The over-winter survival rates were higher in the E2 treatments than in RW. Nineteen percent of hatchlings survived from the RW, 28% from LE2 and 25% from HE2 (Table 31). However, between March and April 2007, a large percentage (49%) of the snails exposed to HE2 during development died (compared to 29% of the LE2 and 20% of the RW F1 snails). F1 snails exposed to HE2 during development were on average smaller than LE2 or RW F1s. F1s from the LE2 treatment were larger, and had the highest percentage of snails in the largest size class, compared to other treatments (Table 32).

Table 31. Survival of F1 *P. corneus* left to depurate in river water over winter, following developmental exposure to river water only (RW), river water plus a low concentration of E2 (LE2) or river water plus a high concentration of E2 (HE2). Numbers left in each treatment tank in Autumn, number surviving until March 2007 and percentage survival over winter.

F1 *P. corneus*	River water (RW)	Low E2 (LE2)	High E2 (HE2)
Left to over-winter September 2006	322	240	442
Surviving March 2007	60	68	109
Percent survival	19%	28%	25%

Table 32. Percentage of different shell size classes represented among all surviving F1 *P. corneus*, after developmental exposure to RW, LE2 or HE2 followed by eight months depuration in river water over autumn/winter 2006. F1 snails were measured in April 2007.

Percentage Shell Size class	River water developmentally exposed	Low E2 developmentally exposed	High E2 developmentally exposed
<10 mm shell diameter	10%	6%	21%
10-15 mm shell diameter	31%	21%	50%
15-20 mm shell diameter	52%	56%	27%
> 20 mm shell diameter	6%	17%	2%

Survival over the 14 week breeding study was lowest (33%) in the snails exposed to HE2 during development and highest (94%) in the LE2 treatment, compared to 44% survival of F1s in the RW treatment. There were significant differences between LE2 and RW/HE2 from week 2 onwards.

No significant differences were found between treatment groups, in terms of F1 snail shell diameters or weights, at the start or finish of the F1 breeding study. There were no significant differences between the mean shell diameter, or weight, of each reproductive group (three per treatment). Over the 14 week study, the mean shell diameter and weight increased by 10.8% and 25.9%, 10.9% and 25.3% and, 11.7% and 32.7% in the RW, LE2 and HE2 treatments, respectively.

Reproduction

Egg laying was delayed by up to six weeks in snails exposed to LE2 and HE2, compared to those from the RW treatment. Snails exposed to RW during development had started laying egg masses by week 2 (first sampling point). In contrast, no eggs were laid by the HE2 or LE2 groups until week 5 and week 8, respectively (Figure 29). In weeks 2, 5 and 8, there were significant differences (reduced egg production) between snails exposed to E2 during development and snails exposed to RW.

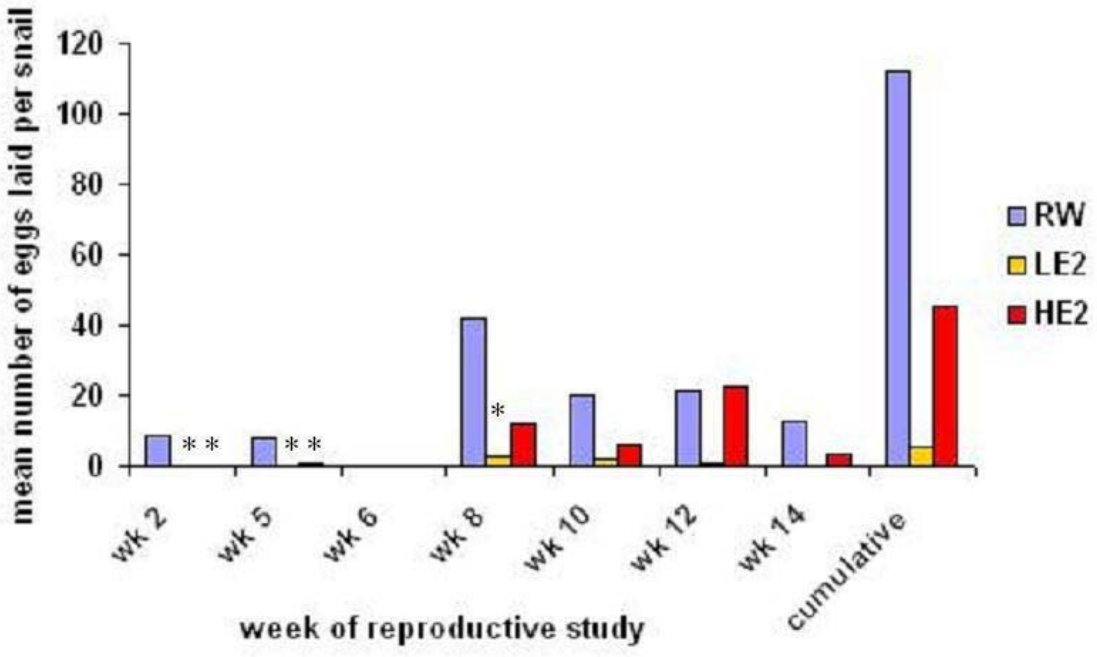

Figure 29. Histogram of mean number of eggs per *P. corneus*. Snails were exposed to RW, LE2 or HE2 during their development and then depurated in river water over autumn/winter 2006. Mean number of eggs laid per snail over each time period during spring/summer 2007 and cumulatively over the entire 14 week study. * indicates a statistically significant difference from RW (P <0.05).

Histopathology of *P.corneus* following depuration

Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail.

Effects on Acini

All snails sampled (from all three treatments) had Sertoli cells attached to the acini walls in all of the sections of gonad examined. A dose-dependent decrease in normal activity in the vitellogenic area (maturing oocytes with follicle cells and/or sperm with Sertoli cells) was found in snails developmentally exposed to E2 and then depurated in river water for approximately one year (Table 35 and Figure 28). Analysis of the average scores, by Oneway ANOVA, found no significant difference between the three treatment groups (P = 0.106).

Table 35. Percentage of F1 *P. corneus* snails with different levels of activity in the vitellogenic area. Snails were examined after exposure to RW, LE2 or HE2 during their development and depuration for approximately one year in river water. The histological analysis was performed on all surviving snails from the F1 breeding study (RW n = 8, LE2 n = 17, HE2 n = 6). Five sections of gonad were analysed per snail.

Percent of acini with active vitellogenic area	Score	Percentage of depurated F1 snails from the river water exposure (RW)	Percentage of depurated F1 snails from the low E2 exposure (LE2)	Percentage of depurated F1 snails from the high E2 exposure (HE2)
<10%	1	0	0	0
10-30%	2	0	6	17
30-50%	3	13	29	33
50-70%	4	49	53	50
>70%	5	38	12	0

The majority of F1 snails (from all treatments) had intact acini walls in all the gonad sections that were examined. Analysis, by Oneway ANOVA, found no significant difference between the three treatments. HE2 treated F1 snails had the least cell coverage (developing oocytes, sperm and supporting cells) on acini walls, while LE2 F1 snails had the most (Table 36). Analysis of the mean scores, by Oneway ANOVA, found no significant difference between the three treatments.

Table 36. Percentage of depurated F1 *P. corneus* snails with different levels of cell coverage on the acini wall. Snails were examined after exposure to RW, LE2 or HE2 during their development and depuration for approximately one year in river water. Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail.

Percent of acini wall covered by germ cells and/or supportive cells	Score	Percentage of depurated F1 snails from the river water exposure (RW)	Percentage of depurated F1 snails from the low E2 exposure (LE2)	Percentage of depurated F1 snails from the high E2 exposure (HE2)
<10%	1	0	0	0
10-30%	2	0	0	0
30-50%	3	13	6	33
50-70%	4	25	24	17
>70%	5	62	70	50

Effects on spermatogenesis

Sertoli cells sloughing into lumen

The number of gonad sections where Sertoli cells were sloughing into the lumen was highest in snails exposed to RW during development and lowest in the snails exposed to HE2 during development. (Table 37). A Oneway ANOVA test found no significant differences between the groups when the mean number of affected sections were compared.

Spermatogonia sloughing into lumen

Sloughing of spermatogonia into the acini lumen was most frequent and least frequent in snails developmentally exposed to LE2 and HE2, respectively (Table 37). However, a Kruskal-Wallis test, which compared the mean number of sections affected by sloughing of spermatogonia, found no significant difference between the groups.

Spermatocyte sloughing into the lumen

The number of sections affected by spermatocytes sloughing into the acini lumen was highest in snails exposed to HE2 during development and lowest in RW snails (Table 37). However, a Oneway ANOVA test found no significant differences between the groups in terms of the mean number of sections affected by sloughing of spermatocyte cells.

Spermatids sloughing into the lumen

The number of sections, with spermatids sloughed into the acini lumen, was highest in snails exposed to RW during development and lowest in the HE2 exposure (Table 37). A Oneway ANOVA test found no significant differences, in the mean number of affected sections, between the groups.

Table 37. Percentage of depurated F1 *P. corneus* snails with different amounts of sloughing of immature spermatogenic cells into the acini lumen. Snails were examined after exposure to RW, LE2 or HE2 during their development and depuration for approximately one year in river water. Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail.

Number of sections affected	Percentage of depurated F1s affected by Sertoli cell sloughed into lumen			Percentage of depurated F1s affected by spermatogonium sloughing into lumen			Percentage of depurated F1s affected by spermatocyte sloughing into lumen			Percentage of depurated F1s affected by spermatid sloughing into lumen		
	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2	RW	LE2	HE2
None	37.5	47	66.6	25	0	50	37.5	35.3	33.3	25	17.6	16.7
1 out of 5	12.5	29	16.6	0	11.8	0	12.5	29.4	0	0	0	16.7
2 out of 5	12.5	12	0	12.5	11.8	0	0	5.9	33.3	12.5	17.6	0
3 out of 5	0	0	16.6	0	5.9	50	12.5	0	0	12.5	17.6	33.3
4 out of 5	25	0	0	25	47.1	0	37.5	17.6	16.7	12.5	17.6	16.7
5 out of 5	12.5	12	0	37.5	23.5	0	0	11.8	16.7	37.5	29.4	16.7

Spermatozoa

The percentage of snails with only mature spermatozoa in the acini lumen was highest in the snails exposed to RW during development and decreased, in a dose-dependent manner, in snails exposed to E2 (Table 38). A Oneway ANOVA test found no significant difference in the mean number of affected sections, between the three groups.

Table 38. Percentage of depurated F1 *P. corneus* snails with different numbers of gonad sections that only contain mature spermatozoa in the acini lumen. Snails were examined after exposure to RW, LE2 or HE2 during their development and depuration for approximately one year in river water. Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail.

No. of sections with just mature spermatozoa in the lumen	Percentage of F1 snails from the river water exposure (RW)	Percentage of F1 snails from the low E2 exposure (LE2)	Percentage of F1 snails from the high E2 exposure (HE2)
None	50	41.2	50
1 out of 5	25	35.3	16.6
2 out of 5	0	11.7	33.3
3 out of 5	0	11.7	0
4 out of 5	0	0	0
5 out of 5	25	0	0

The depurated snails from the LE2 exposure included the highest percentage of snails with the least (<10%) and most (>70%) acini affected by sloughing of immature spermatogenic cells into the lumen. No trend was observed and statistical analysis, by Oneway ANOVA, found no significant differences between the three groups.

Effects on oogenesis

In all three groups the proportions of oocytes at each stage of oogenesis were similar; on average 4% of oocytes were at Stage 5 (mature) (Table 40). Differences were observed between RW and HE2 exposed snails, in terms of the numbers of stage 2 and degenerating oocytes. A Oneway ANOVA test found no significant differences between the groups for any oocyte stage.

Table 40. Stages of oogenesis in depurated F1 *P. corneus* after developmental exposure to RW, LE2 or HE2. Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LE2, n = 17; HE2, n = 6). Five sections of gonad were analysed per snail. Stage 1 (oogonium) is the earliest stage of oogenesis and Stage 5 are ready to be ovulated. The oogenesis stages are based on the description by de Jong-Brink et al. 1979).

Stage of oocyte maturation	Mean % after RW exposure	Mean % after LE2 exposure	Mean % after HE2 exposure
Stage 1	19.9 ± 5.5	25.0 ± 8.2	22.4 ± 11.3
Stage 2	21.8 ± 4.7	18.4 ± 4.3	16.4 ± 3.0
Stage 3	20.0 ± 4.2	20.5 ± 8.3	19.4 ± 3.7
Stage 4	19.9 ± 6.5	16.2 ± 7.2	16.6 ± 7.7
Stage 5	3.6 ± 3.3	4.2 ± 4.2	3.7 ± 3.0
Degenerating	14.8 ± 8.3	15.8 ± 8.5	22.1 ± 5.6

Parasitism

No parasites were observed in any of the F1 snails from the RW, LE2 or HE2 mesocosms.

Effects of E2 developmental exposure on F1 *V. viviparus* growth, survival and development

Survival and growth

At the end of the 16 week adult exposure period the number of neonates (F1s) in the mesocosm tanks was greatest in tanks dosed with E2. The RW tank had 119 neonates, the LE2 tank 175 and the HE2 tank 160. The percentage of snails with a shell length over 10mm (normal minimum size for sexually active snails) was highest (82%) in the RW tank. Seventy-four percent and 63% of the snails in the LE2 and HE2 tanks, respectively, were over 10mm in diameter.

Approximately 40 F1s that were over 10mm in length (and presumed to be sexually mature) were sampled at this point in the experiment: 20 males and 20 females from the RW exposure, 16 females and 25 males from the LE2 exposure and 21 females and 20 males from the HE2 exposure.

Shell length, shell aperture, total weight and soft body weight were analysed. Among the females, there were no significant differences between treatments for shell length ($P = 0.405$), shell aperture ($P = 0.689$) or total weight ($P = 0.162$). There was a significant difference between treatments for soft body weight (shell removed) $P = 0.043$ (Figure 32). Post hoc analysis (LSD) found that the mean soft body weight in HE2 snails was significantly less than the RW snails ($P = 0.017$), but not the LE2 snails ($P = 0.077$). The mean weights of RW and LE2 snails were not significantly different from one another.

Among the males there were significant differences between treatments for shell length ($P = 0.027$), shell aperture ($P = 0.004$), total weight ($P = 0.002$) and soft body weight ($P = 0.002$) (Figure 33). Post hoc analysis (Mann-Whitney test) found that shell length in HE2 snails was significantly shorter than in RW ($P = 0.007$) and LE2 ($P = 0.046$) snails. There was no significant difference between LE2 and RW snails ($P = 0.802$). The shell aperture of HE2 exposed males was significantly smaller than LE2 ($P = 0.008$) and RW snails ($P = 0.002$). There was no significant difference between LE2 and RW snails ($P = 0.973$). Total weight was significantly lower in HE2 compared to RW ($P < 0.001$) and LE2 snails ($P = 0.006$). There was no significant difference between RW and LE2 snails ($P = 0.891$). Soft body weight followed the same trend, HE2 snails were significantly lighter than RW ($P < 0.001$) and LE2 snails ($P = 0.005$). There was no significant difference between RW and LE2 snails ($P = 0.604$).

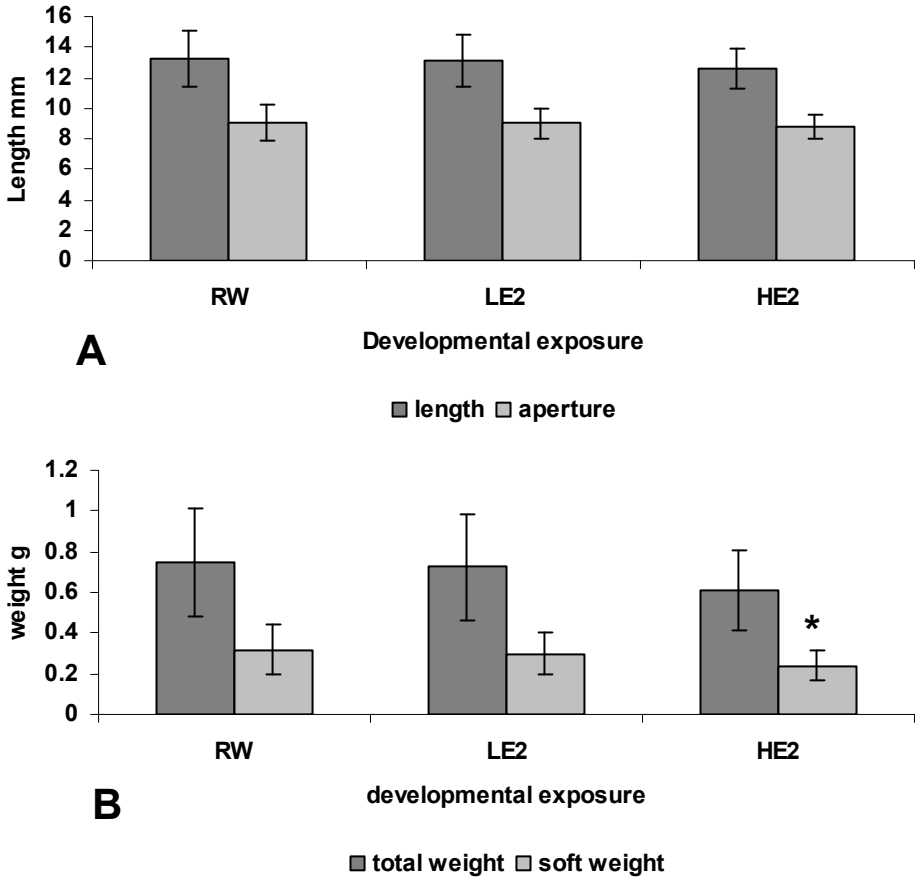

Figure 32. Growth of F1 *V. viviparus* females exposed to RW, LE2 or HE2 during their development. (A) Mean shell length and shell aperture. (B) Mean total weight and soft body weight. RW n= 20, LE2 n= 16 and HE2 n= 21. Error bars give standard error. * indicates a statistically significant difference from RW treatment (P<0.05, LSD).

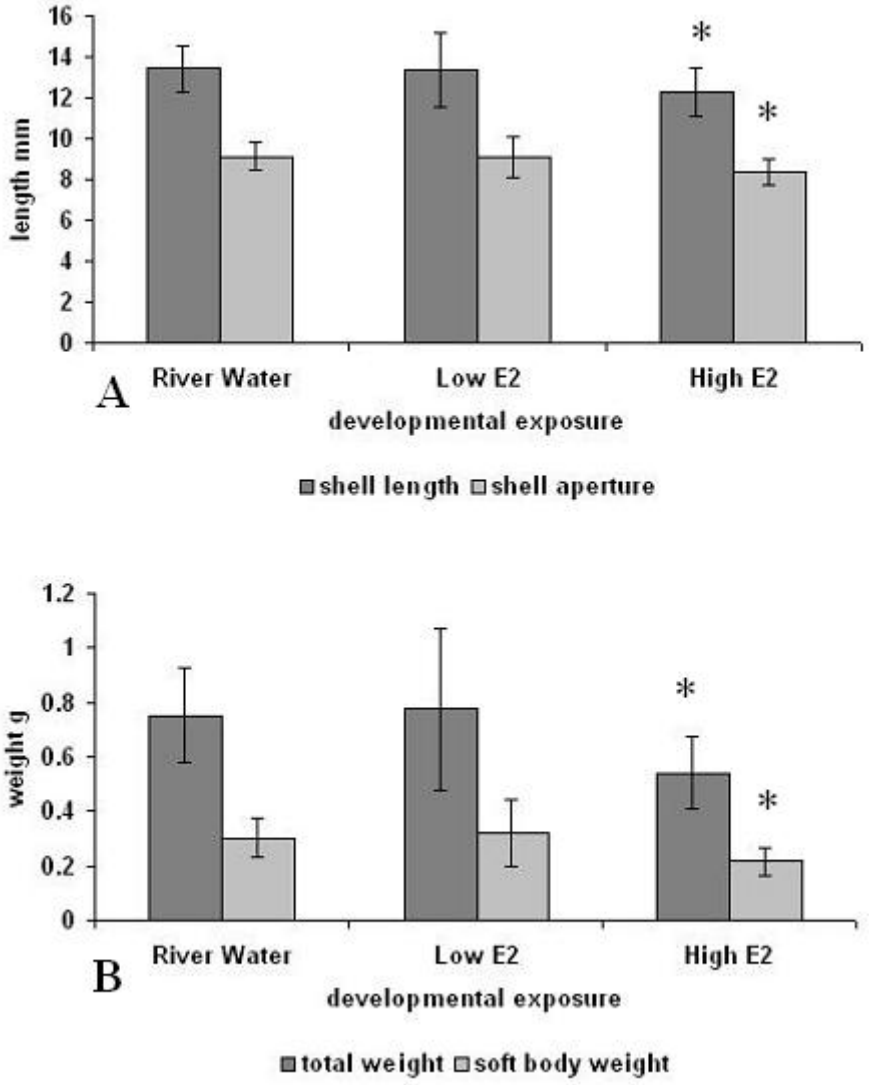

Figure 33. Growth of F1 *V. viviparus* males exposed to RW, LE2 or HE2 during their development. (A) Mean shell length and shell aperture. (B) Mean total weight and soft body weight. RW n= 20, LE2 n= 25 and HE2 n= 20. Error bars give standard error. * indicates a statistically significant difference from other treatment groups (P <0.05, Mann-Whitney).

Histopathology of F1 *V. viviparus* gonads immediately after exposure to E2

After the developmental exposures, samples of approximately 40 F1s were collected from each treatment and examined histologically. Twenty males and 20 females from the RW treatment, 16 females and 25 males from the LE2 treatment, and 21 females and 20 males from the HE2 treatment were examined.

Effects on oogenesis

The mean number of oocytes, per gonad section, decreased in a dose-dependent manner (Figure 34). The females exposed to RW during development had an average of 5.7 oocytes per gonad section, the LE2 females had 2.8 and the HE2 females 2 oocytes per gonad section. A Kruskal-Wallis test identified a significant difference

between the mean oocyte numbers in each treatment (P = 0.002). Post hoc analysis found there was no significant difference between HE2 and LE2 treatments (P = 0.247), but there were significant differences between HE2 and RW (P = 0.001) and LE2 and RW (P = 0.008).

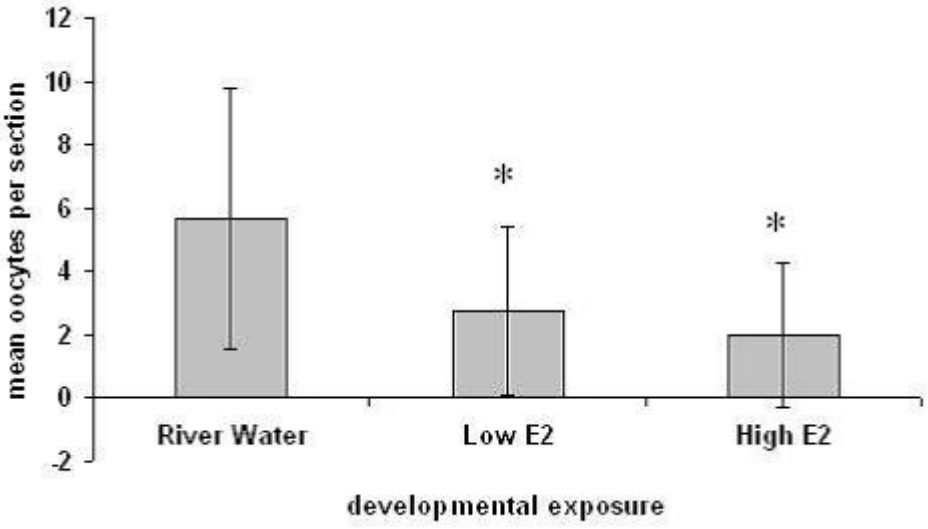

Figure 34. Mean number of oocytes per gonad section of F1 *V. viviparus* females, exposed during development to RW, LE2 or HE2. Samples taken in September 2006. RW, n= 20; LE2, n= 16; HE2, n= 21. Five sections were analysed per female. Error bars give standard deviation. * indicate a statistically significant differences from RW treatment P<0.05 (Mann-Whitney).

Effects on spermatogenesis

The mean score for spermatogenesis decreased in a dose-dependent manner (Figure 35). The males exposed to RW had a mean score of 9 (full spermatogenesis in 50-70% of tubules). LE2 males had a score of 8 (full spermatogenesis in 30-50% of tubules). Males exposed to HE2 had a mean score of 6 (spermatids present). A Kruskal-Wallis test found a significant difference between the treatments (P >0.001). Post hoc analysis, using the Mann-Whitney test, found HE2 snails had a significantly lower score than LE2 (P = 0.003) or RW snails (P <0.001). Snails exposed to LE2 had significantly lower scores than RW snails (P = 0.016).

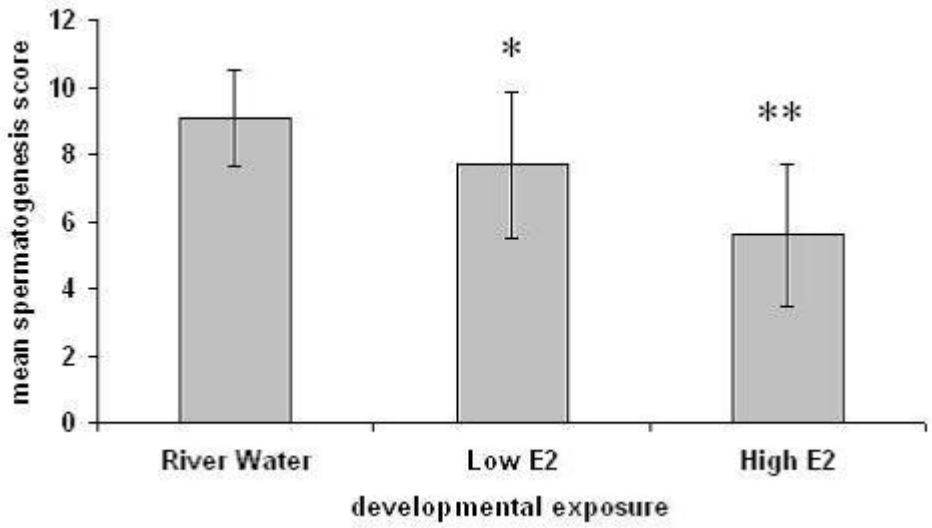

Figure 35. Mean spermatogenesis scores in F1 *V. viviparus* males that had been exposed to RW, LE2 or HE2 during development. Samples taken in September 2006 (RW, n= 20; LE2, n= 25; HE2, n= 20). Score 1 - no germ cells present, score 10 - full spermatogenesis in more than 70% of tubules. Five sections of gonad were analysed per male. Error bars give standard deviation. * indicates a statistically significant difference from RW (P<0.05), ** indicates a statistically significant difference from both RW and LE2 (P<0.05, Mann-Whitney).

Effects of E2 exposure on F1 *V.viviparus* survival, growth, development and reproduction following depuration.

Survival and growth

The percentage survival over winter was higher in the group exposed to LE2 (94%) than the RW (53%) and HE2 (55%) groups (Table 42).

Table 42. Survival of F1 *V. viviparus* left to depurate over winter after developmental exposure to RW, LE2 or HE2. The RW tank contained juveniles from both RW control tanks (E2 and Mixture studies ran in parallel) hence the higher initial number compared to the September count (shown in Table 41).

F1 *V. viviparus*	RW	LE2	HE2
Left to over-winter September 2006	520	125	108
Surviving March 2007	273	117	59
Percent survival	53%	94%	55%

Over the F1 breeding study, survival was highest in the group exposed to LE2 during development (LE2, 97%; HE2, 88%; RW, 85%). At the beginning of the study, the mean shell length (P = 0.929, ANOVA), aperture (P = 0.987, ANOVA) and total weight (P = 0.986, ANOVA) were similar across the treatment groups. However, by the end of the 18 week study, snails exposed to LE2 during development had grown larger than both the RW and HE2 exposed snails. Shell length in the LE2 snails had increased by 24.7%, compared to 16.6% in RW and 18.7% in HE2 snails. The total weight of LE2

exposed snails had increased by 44.5%, compared to 30.6% and 35.8% in the RW and HE2 groups, respectively.

Female snails that had been exposed to LE2 during development were significantly larger than either HE2 or RW exposed snails, both for shell length (RW, P = 0.001; HE2, P = 0.004) and shell aperture (RW, P = 0.005; HE2, P = 0.003). There were no significant differences between RW and HE2 exposed snails. There was a significant difference in mean weight between LE2 and RW female snails (P = 0.007, Figure 56).

In males there were significant differences in shell length between LE2 and RW (P = 0.023) and LE2 and HE2 (P = 0.021) snails, but not between RW and HE2 snails (P = 0.694). There were significant differences in total weight between LE2 and RW (P = 0.021) and LE2 and HE2 (P = 0.036) snails, but not between HE2 and RW snails (P = 0.496) (Figure 56).

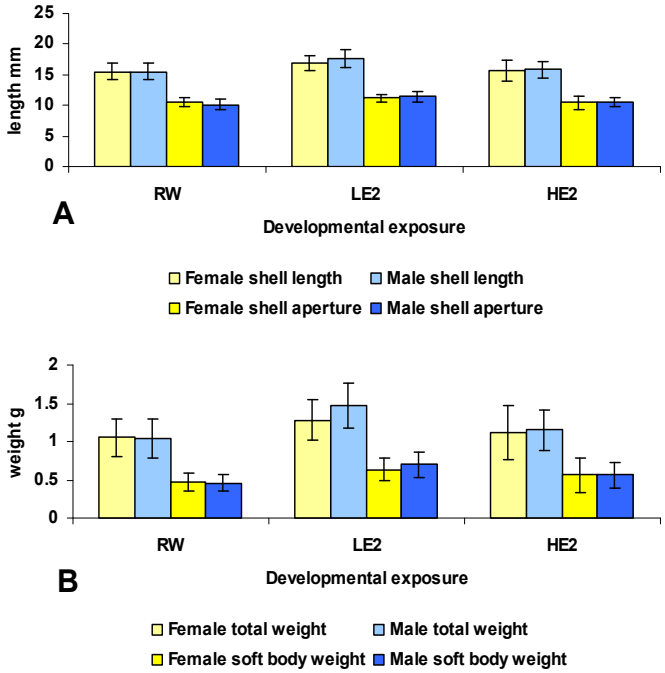

Figure 36. Size of *V. viviparus* F1 snails (males and females) exposed during development to RW, LE2 or HE2 and then depurated in river water at end of the 18-week breeding study. RW, n = 4 males and 24 females; LE2, n = 7 males and 25 females; HE2 n = 8 males and 20 females.

Reproduction

None of the F1 females from any of the three treatments released any neonates during the 18 week breeding study. At the end of the study the percentage of F1 females carrying embryos was highest in the LE2 exposure group; RW 12.5%, LE2 36% and HE2 5%. Females exposed to HE2 during development had significantly fewer embryos, compared to snails exposed to LE2 (total embryos P = 0.008, shelled P = 0.018, unshelled P = 0.009). Females exposed to LE2 had significantly more embryos than snails exposed to RW (total embryos P = 0.037, shelled P = 0.037). There was no significant difference between HE2 and RW snails (total embryos P = 0.369, shelled P = 0.361, unshelled P = 0.369). There was no significant difference in the number of unshelled embryos between RW and LE2 (P = 0.052) (Table 43).

Table 43. Mean number of embryos (shelled or unshelled) present in the brood pouch per F1 female *V. viviparus*. Snails previously exposed to RW, LE2 or HE2 during development then depurated over winter (for 8 months) at the end of the 18 week F1 breeding study. n=21 snails per group. Shared letters (a,b) denote no statistically significant difference between these groups.

	Shelled embryos	Unshelled embryos	Total Embryos
River water developmental exposed	0.04 a	0.33 ab	0.37 a
Low E2 developmental exposed	0.56 b	0.80 b	1.36 b
High E2 developmental exposed	0.00 a	0.05 a	0.05 a

Histopathology of F1 *V. viviparus* gonads following depuration

All surviving F1 snails were examined histologically at the culmination of the F1 breeding study, after approximately one year of depuration. Four males and 24 females from the RW exposure, 25 females and 7 males from the LE2 exposure and 20 females and 8 males from the HE2 exposure, were analysed. Five sections of gonad were analysed per snail.

Effects on oogenesis

The mean oocyte number per snail was highest in snails that had been exposed to RW during their development. Snails exposed to HE2 and LE2 had similar mean numbers of oocytes (Table 47). A Kruskal-Wallis test found a significant difference between treatments (P = 0.041). Post hoc analysis, using the Mann-Whitney test, found a significant difference between RW and LE2 (P = 0.018), but no significant difference between the RW and HE2 (P = 0.247) or LE2 and HE2 (P = 0.117) treatments.

Table 47. Mean oocyte number (±SD) in *V. viviparus* F1 female snails that had been exposed to RW, LE2 or HE2 during development and then depurated in river water for approximately one year. RW, n= 24; LE2, n= 25; HE2, n= 20.

	River water (RW)	Low E2 (LE2)	High E2 (HE2)
Mean oocyte number	4.0 ± 4.06 a	2.0 ± 2.70 b	1.7 ± 1.68 a, b

Effects on spermatogenesis

The mean spermatogenesis score was highest in *V. viviparus* snails that had been exposed to LE2 during development and then depurated for a year (Table 48). There were no significant differences between treatments (P= 0.371, Kruskal-Wallis).

Table 48. Spermatogenesis scores in male *V. viviparus* F1 snails that had been exposed to river water (RW), low concentration of E2 (LE2) or high concentration of E2 (HE2) during development and then depurated in river water for approximately one year. Mean spermatogenesis score (1= least developed, 10 = most developed). RW, n= 4; LE2, n= 7; HE2, n= 8.

Mean spermatogenesis score	River Water (RW)	Low E2 (LE2)	High E2 (HE2)
	9.95 ± 0.1	10.0 ± 0	9.88 ± 0.2

Parasitism

None of the F1 *V. viviparus* from the F1 breeding study were parasitised at any sampling point.

2.4.3 Discussion

The lower overall survival rate in F1 *P. corneus*, compared to F1 *V. viviparus*, is likely to be due to differences in their life strategies. *V. viviparus* has low fecundity, compared to *P. corneus*. Instead of producing a large number of young, *V. viviparus* invests its energy into producing a small number of larger, well developed offspring. These offspring are better equipped to survive the winter. High mortality rates were observed prior to their first reproductive cycle in *P. corneus* that had been exposed to E2 during their development. The reason for this is unknown, but it may be a latent toxic effect of E2 exposure. *V. viviparus* snails did not experience such high mortality, but snails that had been exposed to HE2 were much smaller than the RW (control) snails, this suggests a toxic effect of E2 was retarding their growth. Following depuration, there was, in both species, enhanced survival and growth in the LE2 groups relative to the control population. *V.viviparus* males were, on average, larger than the females. This is contrary to the published literature, where reports state that males of this species are smaller than the females (Jezewski, 2004; Jakubik, 2003, 2006 and 2007). As the size of the male sample in this study was quite small the findings may be a sampling artefact.

P. corneus offspring, exposed to E2 during development, exhibited a significant dose-dependent reduction in activity in the vitellogenic area (RW>LE2>HE2). This was similar to, but more significant than, the response seen in their parents. Significant differences were found between the two E2 exposure groups as well as between the RW and E2 exposures. Sloughing of immature spermatogenic or Sertoli cells, into the acini lumen, was most frequent in snails exposed to E2. The percentage of lumen containing sloughed spermatogenic cells was significantly higher in both the E2 groups than in the RW treatment. The number of gonad sections that contained only mature spermatozoa in the acini lumen was significantly reduced in snails exposed to HE2 during development.

Developmental exposure to E2 affected the proportions of different stages of oogenesis present in F1 *P. corneus*. The effect was similar to that seen in exposed adults, but more severe. The percentages of young oocytes (stages 1 and 2) were significantly higher in LE2 and HE2 treated snails, than snails exposed to RW. Snails exposed to HE2 had significantly fewer stage 4 oocytes, and both HE2 and LE2 treatments had significantly fewer degenerating oocytes. This suggests E2 has an effect on oocyte maturation (possibly mediated via the follicle cells). There are several ways the follicle cells may have been affected, leading to a reduction in mature oocytes in the vitellogenic area. Firstly, the ability of the follicle cells to transport the developing

oocytes to the vitellogenic area may have been inhibited, thus promoting empty vitellogenic areas. Secondly, the ability of the follicle cells to support oocyte maturation (which normally occurs in the vitellogenic area) may have been disrupted. In vertebrates, there has been less research on the effects of E2 on female germs cells than its effects on male germ cells. However, it has been reported that, in sexually mature fathead minnows exposed to E2 (0.0625-1000nM), there were increased numbers of primary stage oocytes and reduced numbers of later stage oocytes (Miles-Richardson et al. 1999). Therefore, the reduced activity in the vitellogenic area could be due to the combined effects of Sertoli cell disruption (and sloughing) and a reduction in oocyte maturation.

Effects on spermatogenesis and oogenesis were also found in *V. viviparus* exposed to E2 during development. F1 females, from both E2 exposures, had significantly fewer oocytes per gonad section than snails exposed to RW. The male spermatogenesis score (sexual development) was significantly affected, in a dose-dependent manner (even after accounting for differences in the size of the snails). The males exposed to HE2 only had spermatids present in the gonad, compared to full spermatogenesis in 50-70% of tubules in gonad sections from males exposed to RW. Sertoli cells were not seen to detach from the tubule basement membrane (as was seen in *P. corneus*), but this retarded spermatogenesis might still be evidence of Sertoli cell dysfunction. Developmental exposure to steroid oestrogens has been reported to disrupt normal spermatogenesis in vertebrates. For example, exposing 1 day old rats to E2 (1μg) resulted in altered gonocyte migration, reduced cell proliferation and increased apoptosis (Vigueras-Villasenor et al. 2006). Atanassova et al. (1999) reported similar results (reduced germ and Sertoli cell volume (per testis) in adults and increased apoptosis) when they exposed developing rats to 10μg/kg EE2.

Further evidence that the snails exposed to E2 had been subject to a form of endocrine disruption was seen during the breeding study. Sexual maturation was delayed in *V. viviparus* across all treatments, but fewer embryos and developing oocytes were found in the E2 treated tanks than in the reference tanks. Similarly, in *P. corneus*, egg laying was delayed in both LE2 and HE2 treated snails, relative to the controls. During the short time they did lay eggs, the LE2 snails laid at a much lower rate than the controls. In *P. corneus,* as in other Basommatophora, egg laying is regulated by both biotic and abiotic factors; the most significant of these are day length, water temperature and nutritional state (Scheerboom, 1978; Bohlken and Joosse, 1982; Joosse, 1984; Costil and Daguzan, 1995a, b). In experiments with another UK native pulmonate, *Lymnaea stagnalis*, day length was found to be the overriding external factor in controlling reproductive output (Bohlken and Joosse, 1982). *P. corneus* normally start laying eggs in early spring and continue until late summer (Costil and Daguzan, 1995b; Clarke et al. 2009). However, the snails exposed to E2 during their development did not start laying eggs until around week 8 of the study. The summer solstice (longest day) fell between weeks 8 and 10, when maximum reproductive output is normally achieved.

The absence of embryos in some female *V. viviparus*, across all treatments, was somewhat unexpected. Jakubik (2007) reported that female *V. viviparus* from the Zegrzynski Reservoir (Central Poland) had between 0.9 and 6.7 embryos per female (at a shell length of 8.1 - 12mm) and 1.1 - 9.6 embryos per female (at a shell length of 12.1 - 25mm). The low embryo numbers may be density related. The densities of juvenile snails were 0.8l/snail in the RW tank, 1.8l/snail in the LE2 tank and 1.2l/snail in the HE2 tank. *V. viviparus* are known to aggregate at extremely high densities under good conditions. For example, Jakubik (2003) reported densities of over 800 individuals/m^2 in the Zegrzynski Reservoir. However, Browne and Richardson (1992) have reported that these aggregations are not observed when food sources are limited. Food supplies may have been restricted during the over-wintering period as the river water pump had to be removed from the river and switched off (leaving the tanks

static). This might have reduced the food supply and dissolved oxygen levels (although the aeration pump was still active). Increased competition for food and resources (especially in the RW tank) may have hampered reproduction and compromised the snails' ability to produce eggs. The over wintering of snails in these mesocosm tanks had not been attempted before and the carrying capacity of the tanks was unknown. The results of this experiment suggest that 100 F1 *V. viviparus* per tank would, perhaps, be optimum for over-wintering survival.

The observation that, at the end of the experiment, significantly fewer embryos were carried by depurated females that had been exposed to HE2, compared to females exposed to LE2, is less likely to be an effect of snail density as population densities in the two treatments were similar. The reduced oocyte numbers, combined with lower spermatogenic scores, seen in *V. viviparus* F1s post HE2 exposure are important. Due to the long gestation period in this species, mating must occur in a female's first autumn for her to release offspring the following spring/summer. In this experiment, males exposed to HE2 were not (on average) producing any mature sperm in September. This would have prevented many of the snails from successful mating. After a year of depuration in river water, oocyte numbers were still subject to a dose-dependent decrease in production, while in depurated males spermatogenesis had fully recovered. It seems likely that in developing *V. viviparus,* the effects of E2 on spermatogenesis were transient while the effects on oocyte numbers were more permanent. This effect may have a significant impact on this long lived species, as a short exposure to E2 at the developmental stage could seriously affect recruitment for an extended period. It may have been useful to extend this experiment to fully appreciate the long term effects of E2 exposure. For example, Kidd et al (2007) reported that chronic exposure to the synthetic oestrogen EE2 (5-6ng/l) led to near extinction of fathead minnows (*P. promelas*) in an experimental lake in Canada. Early signs of the effects of EE2 on these fish included retarded spermatogenesis in males and altered oogenesis in females, similar findings to those observed in *V. viviparus* exposed to E2.

2.5 Mesocosm experiment 3a: Exposure of two UK native freshwater gastropod species to a mixture of chemicals in an outdoor mesocosm

2.5.1 Methods

Experimental set up

This experiment, on the effects of exposure to a mixture of EDCs, was run in parallel with the E2 exposure experiment (experiment 2). It had its own river water (RW) control, a river water plus ethanol treatment (solvent tank, RW+S) and river water plus EDC mixture at low (LM) and high (HM) concentrations. All other aspects of the experimental set-up and sampling regime were the same as experiment 2.

The EDC mixture included environmentally relevant concentrations of estrone (E1), estradiol (E2), ethinylestradiol (EE2), nonylphenol (NP), nonylphenol ethoxylates (NP1EO and NP2EO) octylphenol (OP), and Bisphenol A (BPA). River Chelmer water was used as the diluent. To simulate a typical effluent stream, nominal concentrations of EDCs in the HM tank were 100ng/l E1, 10ng/l E2, 1.5ng/l EE2, 6000ng/l NP,

5000ng/l NP1EO, 7000ng/l NP2EO, 600ng/l OP and 100ng/l BPA. To simulate a typical river the nominal concentrations in the LM tank were 20ng/l E1, 3ng/l E2, 0.5ng/l EE2, 1500ng/l NP, 1000ng/l NP1EO, 2000ng/l NP2EO, 150ng/l OP and 50ng/l BPA. Control tanks included River Chelmer water plus ethanol solvent (dosed at 0.5 μl/l) and a tank with River Chelmer water alone.

Test organisms

Adult snails, six *P.corneus* or eleven *V.viviparus*, were randomly designated to each replicate cage. There were six replicate cages per species, per tank. The *P. corneus* were fed twice a week on approximately 30g organic carrot and approximately 30g flake fish food per cage.

Chemical analysis

APs and APEOs

Aqueous samples were solvent extracted using 50/50 v/v diethyl ether/iso-hexane. The extracts were concentrated before GC-MS with EI detection (operated in SIM mode). The calibration standards were a technical mix of isomers for NP, pure p-tert OP, a mix of isomers for NP-ethoxylates and a single isomer for OP-ethoxylate. The minimum reportable values (MRV) for NP and OP were 100ng/l and 200ng/l, respectively.

BPA

Samples were spiked with deuterated BPA before solvent extraction with DCM. Extracts were dried before derivatisation using trifluoroacetic anhydride. The same GC-MS system was used as for APEs. The minimum reportable value (MRV) for BPA was 40ng/l.

Steroid Estrogens

As described for experiment 1. Minimum reportable values (MRVs) for E2, E1 and EE2 were 0.3 ng/l, 1 ng/l and 0.1 ng/l, respectively.

Yeast screen

Samples for the YES assay were taken from the two mixture tanks (HM and LM) and the solvent tank (RW+S) at weeks 2, 6, 8, 10, 12 and 16. Samples were taken from the river water only tanks at weeks 8, 10, 12 and 16.

2.5.2 Results

Chemical analysis

The measured concentrations of steroids and EDCs in the tanks at each sampling point are given in Table 50. The RW and RW+S tanks had similar mean E1 concentrations of 1.22 ± 0.46ng/l and 1.37 ± 0.63ng/l, respectively. The LM tank had a mean E1 concentration of 30.96 ± 48.04ng/l and the HM tank had the highest E1 mean concentration of 60.54 ± 61.01ng/l. The E2 concentrations in the RW and RW+S tanks were 3.34 ± 4.0ng/l and 1.71 ± 2.7ng/l, respectively. E2 concentrations in the LM and HM tanks were similar, 10.87 ± 7.84ng/l and 11.28 ± 11.46ng/l, respectively. High concentrations of EE2 were found in the river water pump inlet at weeks 12 and 16, with values up to 3.73ng/l. High EE2 concentrations were also measured in the RW and RW+S tanks; 1.11 ± 0.94ng/l and 1.18 ± 0.91ng/l, respectively. EE2

concentrations in the LM and HM tanks were similar, 2.87 ± 1.6ng/l and 3.26 ± 2.4ng/l, respectively.

The measured concentrations of BPA were 0.08 ± 0.08μg/l and 0.12 ± 0.1μg/l in the RW+S and RW tanks, respectively. BPA concentrations in the LM and HM tanks were similar, 0.27 ± 0.4μg/l and 0.33 ± 0.3μg/l, respectively. The NP concentration in both the RW and RW+S tanks was 0.34 ± 0.22μg/l. The concentration in the LM tank was 0.42 μg/l, while that in the HM tank was higher at 1.47 ± 1.67μg/l. None of the water samples taken from any of the tanks had measured concentrations of OP above the MRV.

Table 50. Mean measured concentration (± SD) of steroid and non-steroid estrogens from the RW, RW+S, LM, HM tanks and river water pump inlet (river water which fed all tanks) over a 16 week dosing period. Mean concentrations of E2, E1 and EE2 are measured in nanograms per litre (ng/l). Mean concentration of non-steroid estrogens (NP, BPA, and OP) measured in micrograms per litre (μg/l). Water chemistry provided by Environment Agency's National Laboratory Service, Nottingham.

Tank	E1 (ng/l)	E2 (ng/l)	EE2 (ng/l)	NP (μg/l)	BPA (μg/l)	OP (μg/l)
RW	1.22 ± 0.5	3.34 ± 4.0	1.11 ± 0.9	0.34 ± 0.2	0.12 ± 0.1	0.68 ± 0.4
RW+S	1.37 ± 0.6	1.71 ± 2.7	1.18 ± 0.9	0.34 ± 0.2	0.08 ± 0.1	0.68 ± 0.4
LM	30.96 ± 48.0	11.28 ± 11.5	2.87 ± 1.6	0.42 ± 0.2	0.27 ± 0.4	0.68 ± 0.4
HM	60.54 ± 61.0	10.87 ± 7.8	3.26 ± 2.4	1.47 ± 1.7	0.33 ± 0.3	0.68 ± 0.4
Inlet	1.3 ± 0.2	0.34 ± 0.1	1.70 ± 1.8	not measured	not measured	not measured

Yeast screen

Water samples taken from the HM tank ranged from <0.87 - 27.27ng/l E2 EQ (mean 9.29ng/l E2 EQ). Samples from the LM tank ranged from <0.85 - 80.57ng/l E2 EQ (mean 16.05ng/l E2 EQ). Samples from the RW tank ranged between 0 - 1.75ng/l E2 EQ (mean 1.03ng/l E2 EQ) and the RW+S tank ranged between <0.475 - 10.6ng/l E2 EQ (mean 0.75ng/l E2 EQ). The river water inlet ranged between 0 - 2.01ng/l E2 EQ (mean 0.88ng/l E2 EQ) (Table 51). The E2 EQ was found to be significantly higher in the LM and HM tanks, compared to the RW ($P = 0.032$) or RW+S tanks ($P = 0.009$).

Table 51. Results of yeast estrogen screen (YES) assay measurements of mean E2 EQ ng/l (± SD) for each mesocosm tank (RW, RW+S, LM, HM) and the river water inlet form. No water samples were taken from the RW tank or river inlet at weeks 2 and 6. Water samples taken at weeks 8, 12 and 16 were taken simultaneously for both YES assay and chemical analysis (highlighted).

	Week 2	Week 6	Week 8	Week 10	Week 12	Week 16	mean
RW	-	-	0	1.67	0	1.75	0.86 ± 0.99
RW+S	1.06	0	0	0	0	0.89	0.33 ± 0.51
LM	3.25	1.96	80.57	5.84	0.85	3.83	16.05 ± 31.66
HM	9.25	3.28	11.77	3.31	27.27	0.87	9.29 ± 9.72
inlet	-	-	2.01	0	0	0.95	0.74 ± 0.96

Effects of exposure to estrogenic mixtures on survival, growth and reproduction of adult *P. corneus*

Survival

Until week 10, adult snail survival was lower in snails exposed to RW than in groups exposed to RW+S, LM and HM (Figure 41). After week 10, snails exposed to RW+S suffered relatively high mortalities. There were 25 surviving snails in each of the RW, LM and HM groups at the end of the 16 week exposure, from an original population of 36. In the RW+S tank only 5, out of 36, snails survived.

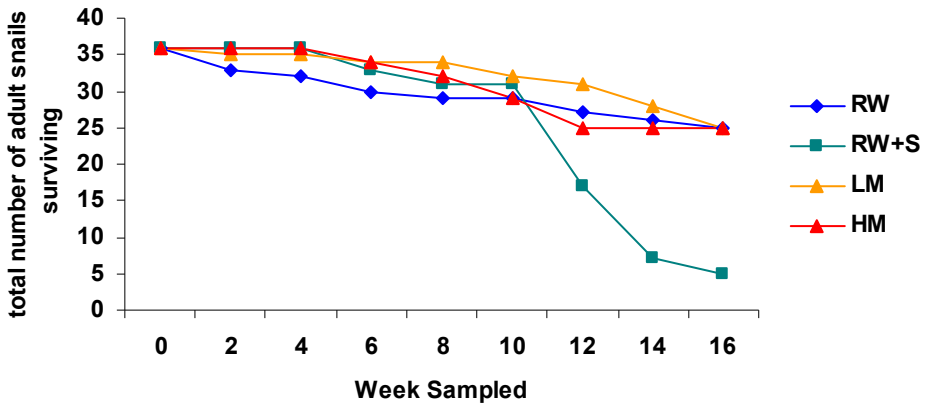

Figure 41. Total number of surviving adult *P. corneus* in each treatment, counted at each fortnightly sampling point over the 16 week breeding study exposure period. Six groups of 6 *P. corneus* snails (n = 36/treatment) were exposed to RW, RW+S, LM or HM.

Growth

At the start of the experiment the mean shell sizes (diameter and aperture) were similar across all treatment groups. The mean total weight was also similar between groups and ranged from 1.8 to 2.0 grams. A Oneway ANOVA test found no significant differences between the four treatment groups for shell diameter (P = 0.633), shell aperture (P = 0.505) or total weight (P = 0.575).

Snails in all the treatments grew over the 16 week exposure. A Oneway ANOVA test found no significant differences between the four treatment groups after 16 weeks of exposure. Growth rates were highest in the snails exposed to RW+S, but mortality was also high in this group (see above). The next highest growth rate was in snails exposed to HM. These snails grew by 14.8% in shell diameter and 30.7% in total weight, on average, (Table 52) compared to the 9.1% increase in shell diameter and 21.4% increase in total weight seen in snails exposed to RW.

Table 52. Size of adult *P. corneus* at allocation (start of experiment) and after 16 weeks of exposure to RW, RW+S, LM or HM. Mean shell length and total weight (±SD) are given plus mean percentage growth over the 16 weeks.

	River water (RW)	River water plus Solvent (RW+S)	Low mixture (LM)	High mixture (HM)
START Shell diameter mm	22.1 ± 2.2 mm	21.9 ± 2.1 mm	22.5 ± 2.7 mm	22.0 ± 2.1 mm
FINISH Shell diameter mm	24.3 ± 2.1 mm	25.9 ± 2.2 mm	25.4 ±1.6 mm	25.8 ± 2.3 mm
Growth %	9.1%	15.7%	11.2%	14.8%
START Total weight g	1.9 ± 0.6 g	1.9 ± 0.5 g	2.0 ± 0.7 g	1.8 ± 0.5 g
FINISH Total weight g	2.4 ± 0.6 g	2.8 ± 0.9 g	2.5 ± 0.6 g	2.6 ± 0.7 g
Growth %	21.4%	33.7%	20.2%	30.7%

Reproduction

The mean numbers of eggs laid per snail per fortnight were similar between the four exposure groups at each sampling point. There were no statistically significant differences, between the four groups, in the mean numbers of eggs laid per snail.

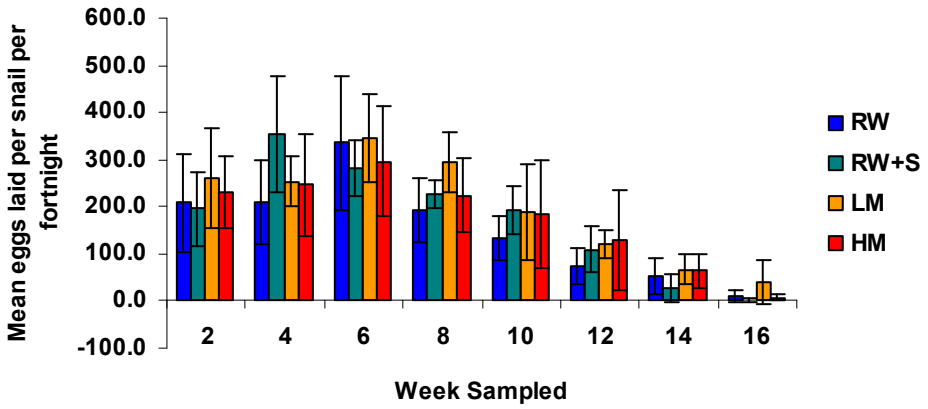

Figure 42. Mean number of eggs laid (±SD) per adult *P. corneus*, per fortnight, during exposure to RW, RW+S, LM or HM.

Histopathology of adult *P. corneus* exposed to an estrogenic mixture

Histological analysis was performed on a sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18). Four sections of gonad were analysed per adult snail. Due to the complex nature of the *P. corneus* gonad, the analysis was split between different areas, or cell types, within the gonad.

Effects on Acini

All the gonad sections from all the snails in the RW and RW+S treatments had Sertoli cells attached to the acini walls. One snail from the LM treatment had one gonad section (of four) where there were no Sertoli cells attached to the acini walls. In one snail from the HM treatment, there were no Sertoli cells attached to the acini walls in any of the four gonad sections. There was a dose-dependent decrease in normal activity in the vitellogenic area (maturing oocytes with follicle cells and/or sperm with Sertoli cells) (see Table 53). However, this reduction in activity was not statistically significant P = 0.539 (Kruskal-Wallis test).

Table 53. Percentage of adult *P. corneus* snails with different levels of activity in the vitellogenic area. Snails examined after four months exposure to RW, RW+S, LM or HM. Histological analysis was performed on a sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18 (one parasitised individual was omitted)). Four sections of gonad were analysed per adult snail.

Percent of acini with active vitellogenic area	Score	Percentage of adult snails from the RW exposure	Percentage of adult snails from the RW+S exposure	Percentage of adult snails from the LM exposure	Percentage of adult snails from the HM exposure
<10%	1	0	0	7%	0
10-30%	2	5 %	0	0	0
30-50%	3	0	0	0	18%
50-70%	4	45%	40%	50%	53%
>70%	5	50%	60%	43%	29%

Adult snails from the RW+S exposure had the greatest cell coverage (developing oocytes, sperm and supporting cells) on the acini walls. Adult snails from the HM

treatment had the least cell coverage (Table 54). There was a significant difference, between the four treatment groups, for acini wall coverage (P = 0.010, ANOVA). Post hoc analysis found that snails exposed to HM had significantly less coverage of acini walls than snails exposed to RW (P = 0.003, LSD) or RW+S (P = 0.011).

Table 54. Percentage of adult *P. corneus* snails with different degrees of cell coverage of the acini wall. Snails examined after four months exposure to RW, RW+S, LM or HM. Histological analysis was performed on a sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18 (one parasitised individual was omitted)). Four sections of gonad were analysed per adult snail. Shared letters (a, b) indicate there is no statistically significant difference between those groups.

		% of adult snails			
Percent of acini wall covered by germ cells and/or supportive cells	**Score**	**RW**	**RW+S**	**LM**	**HM**
<10%	1	0	0	0	6%
10-30%	2	5%	0	29%	12%
30-50%	3	20%	0	0	35%
50-70%	4	15%	20%	29%	35%
>70%	5	60%	80%	42%	12%
		a	a	ab	b

Acini wall disruption was found in only one snail, this was a heavily parasitised individual from the HM treatment. The disruption appeared to be due to the parasitic infestation. All other adult *P. corneus*, from all treatments, had intact acini walls.

Effects on spermatogenesis

Sertoli cells sloughing into lumen

The adult snails from the RW+S treatment had the fewest gonad sections where Sertoli cells were found to be sloughing into the acini lumen. Snails from the HM treatment had the highest level of sloughing (Table 55). There was a significant difference between the four treatment groups (P = 0.015 Kruskal-Wallis test). Post hoc analysis found that snails exposed to RW had significantly less sloughing of Sertoli cells, compared to the LM (P = 0.050 M-W) and HM (P = 0.004) snails. There was no significant difference between snails exposed to RW+S and RW (P = 0.617).

Spermatogonium sloughing into lumen

Sloughing of spermatogonia cells into the acini lumen was found most frequently in snails exposed to LM and least frequently in snails exposed to RW+S (Table 55). However, there was no statistically significant difference between the four treatment groups (P = 0.477, Kruskal-Wallis test).

Spermatids sloughing into the lumen

Sloughing of spermatids into the lumen was found most frequently in snails exposed to LM and least frequently in snails exposed to RW+S (Table 55). There was no significant difference between the four treatments (P = 0.531, ANOVA).

Spermatocyte sloughing into the lumen

Sloughing of spermatocytes into the lumen was found most frequently in snails exposed to HM and least frequently in snails exposed to RW+S (Table 55). No significant difference was found between the four treatment groups (P = 0.063, Kruskal-Wallis test).

Table 55. The percentage of adult *P. corneus* snails with different levels of sloughing of immature spermatogenic cells into the acini lumen. Measurements made after four months exposure to RW, RW+S, LM or HM. Histological analysis was performed on a sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18 (one parasitised individual omitted)). Four gonad sections were analysed per adult snail. Shared letters (a, b) indicate no statistically significant difference between these groups.

	% of adult *P. corneus* with spermatogenic cells sloughed into lumen															
Number of sections affected	**Sertoli cells sloughed into lumen**				**Spermatogonia sloughed into lumen**				**Spermatocytes sloughed into lumen**				**Spermatids sloughed into lumen**			
	RW	RW+S	LM	HM	RW	RW+S	LM	HM	RW	RW+S	LM	HM	RW	RW+S	LM	HM
None	95	100	71.4	52.9	30	20	7.1	23.5	85	80	50	41.2	35	40	21	29.4
1 out of 4	5	0	7.1	17.6	10	20	0	11.8	0	20	0	11.8	20	20	0	17.6
2 out of 4	0	0	14.3	5.9	5	40	21.4	11.8	0	0	0	11.8	5	20	36	5.9
3 out of 4	0	0	7.1	17.6	10	0	21.4	5.9	0	0	21	5.9	10	20	7.1	29.4
4 out of 4	0	0	0	5.9	45	20	50	47.1	15	0	29	29.4	30	0	36	17.6
	a	ab	B	b	a	a	a	A	A	ab	ab	b	a	a	a	A

Spermatozoa

The adult snails exposed to RW+S had the highest frequency of gonad sections that only contained mature spermatogenic cells in the acini lumen, snails exposed to LM had the lowest frequency (Table 56). There was no significant difference between the four treatment groups (P = 0.120, Kruskal-Wallis test).

Table 56. The percentage of adult *P. corneus* snails with different numbers of gonad sections that only contained mature spermatozoa in the acini lumen. Measurements made after four months exposure to RW, RW+S, LM or HM. Histological analysis was performed on a sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18 (one parasitised individual omitted). Four gonad sections were analysed per adult snail.

	% of adult *P. corneus* with only mature spermatozoa in acini lumen			
No. of sections	**RW**	**RW+S**	**LM**	**HM**
0 out of 4	45%	20%	64%	64.7%
1 out of 4	10%	0	29%	5.9%
2 out of 4	5%	40%	0	0
3 out of 4	15%	20%	0	23.5%
4 out of 4	25%	20%	7%	5.9%

Overall, the percentage of acini (per section of gonad) where immature spermatogenic cells had sloughed into the lumen, was lowest in snails exposed to RW+S and highest in those exposed to LM. There was no significant difference between the four treatment groups (P = 0.581, ANOVA).

Effects on oogenesis

The proportions of different stages in oocyte development, which were present in snail gonads were similar across all treatments (Table 58). The mean percentage of early oocyte stages (1 and 2) was highest in snails exposed to LM. The mean percentage of mature oocytes (stage 5) was highest in snails exposed to RW+S. A Oneway ANOVA test found significant differences between the groups for stage 2 (P = 0.005) and 3 (P = 0.015) oocytes. No significant difference was found for stage 1 (P = 0.140), stage 4 (P = 0.168), stage 5 (P =0.487) or degenerating oocytes (P = 0.057). The results of the post hoc analysis are shown in Table 58. Snails exposed to HM had a significantly lower percentage of stage 2 oocytes, compared to the RW (P = 0.024, LSD) and LM (P >0.001) treatments. The snails exposed to LM had a significantly lower percentage of stage 3 oocytes than the snails exposed to RW (P = 0.001, LSD) or HM (P = 0.026). Snails exposed to RW+S had a significantly higher percentage of stage 4 oocytes, compared to snails exposed to RW (P = 0.040, LSD) and LM (P = 0.043). The HM snails also had a significantly higher percentage of degenerating oocytes than snails exposed to RW (P = 0.036 LSD) or LM (P = 0.013) (Table 58).

Table 58. The mean percentage of different stages in oogenesis, in adult *P. corneus*, after four months exposure to RW, RW+S, LM or HM. Histological analysis was performed on a sample of adults from each treatment (RW, n = 20; RW+S, n = 5; LM, n = 18; HM, n = 18 (one parasitised individual was omitted)). Four gonad sections were analysed per adult snail. Stage 1 (oogonium) is the earliest stage of oogenesis, stage 5 is ready to be ovulated. Oogenesis stages are based on descriptions by de Jong-Brink et al. (1979). Shared letters (a, b) indicate there is no statistically significant difference between these figures.

Stage of oocyte maturation	Different stages of oogenesis (mean %)			
	RW	RW+S	LM	HM
Stage 1	22.4 ± 9.1 a	17.2 ± 3.5 a	26.7 ± 6.5 a	22.6 ± 8.7 a
Stage 2	24.9 ± 4.3 a	24.1 ± 3.8 ab	27.3 ± 3.6 a	21.8 ± 4.2 b
Stage 3	25.8 ± 5.0 a	23.5 ± 2.6 ab	20.4 ± 4.2 b	24.2 ± 4.9 a
Stage 4	12.4 ± 5.1 a	17.7 ± 3.4 b	12.3 ± 4.2 a	13.8 ± 5.7 a
Stage 5	1.5 ± 1.4 a	2.4 ± 3.2 a	1.5 ± 1.6 a	1.1 ± 1.4 a
Degenerating	13.0 ± 5.2 a	15.2 ± 3.1 ab	11.9 ± 4.5 a	16.5 ± 5.3 b

Parasitism

Only one snail had a visible parasitic infection. This was extremely heavy and had eliminated the whole gonad. The infected snail came from the HM treatment.

Effects of exposure to estrogenic mixtures on adult *V. viviparus* survival, growth and reproduction

Survival

During the study, the highest adult survival was in the LM treatment and the lowest was in the RW+S treatment (Figure 44). There were no statistically significant differences between the four treatment groups in terms of the number of adults surviving at any time point.

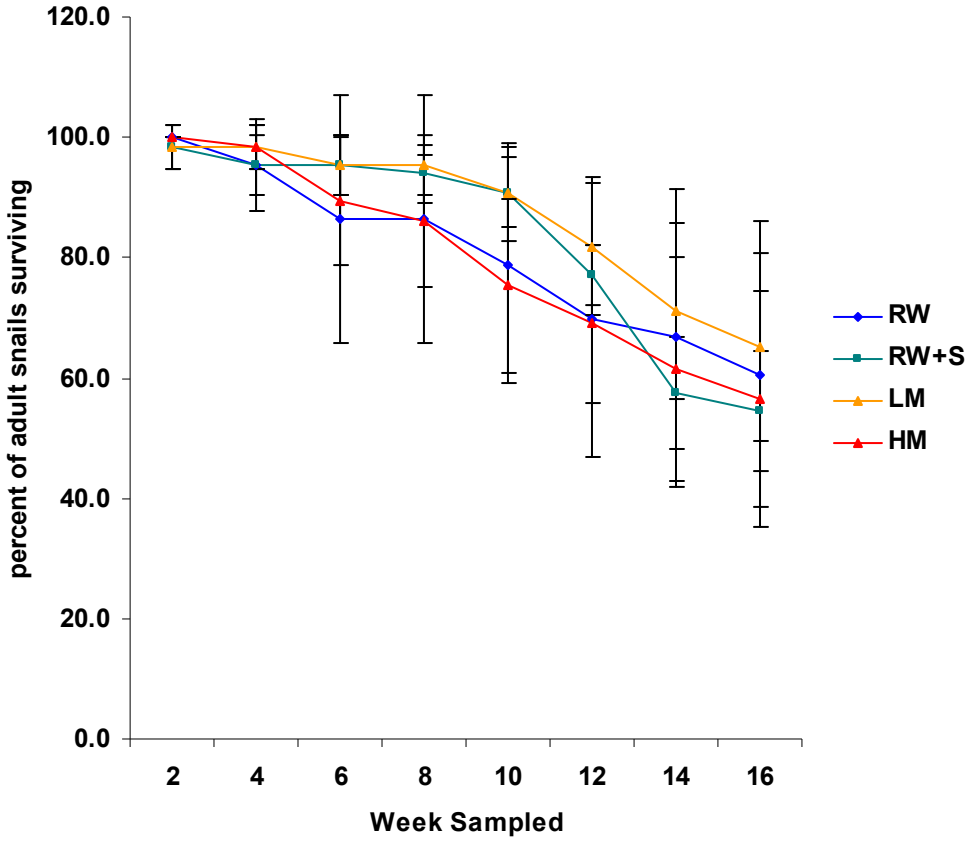

Figure 44. Mean percentage of surviving adult *V. viviparus* (both sexes) over the 16 week exposure to RW, RW+S, LM or HM. Error bars show standard deviation of the mean.

Growth

At the start of the experiment, the mean values for shell length, shell aperture and total weight were similar across the exposure groups. There were no statistically significant differences between the groups in shell length (P = 0.793, ANOVA), shell aperture (P = 0.776, ANOVA) or total weight (P = 0.448, ANOVA).

At the end of the exposure, the mean values for shell length, shell aperture, total weight, soft body weight and soft body weight with embryos removed, for females (Table 60) and males Table (61) were similar over all treatments. There were no statistically significant differences (ANOVA) between the treatment groups.

Table 60. Mean (± SD) shell length, shell aperture, total weight, soft body weight and soft body weight with embryos removed of surviving adult *V. viviparus* females, after four months exposure to RW, RW+S, LM or HM.

Size	RW	RW+S	LM	HM
Shell length mm	33.3 ± 2.2	32.8 ± 4.2	32.4 ± 2.7	32.1 ± 3.0
Shell aperture mm	17.6 ± 1.2	17.4 ± 1.7	17.3 ± 1.3	17.0 ± 1.3
Total weight g	8.5 ± 1.4	8.3 ± 2.2	8.0 ± 1.6	7.8 ± 1.6
Soft body weight g	3.2 ± 0.6	3.2 ± 0.9	3.0 ± 0.7	3.0 ± 0.6
Soft body weight embryos removed g	1.9 ± 0.4	2.0 ± 0.6	1.9 ± 0.5	1.8 ± 0.3

Table 61. Mean (± SD) shell length, shell aperture, total weight and soft body weight of surviving adult *V. viviparus* males, after four months of exposure to RW, RW+S, LM or HM. RW, n= 4; RW+S, n= 6; LM, n= 5; HM, n =2.

	RW	RW+S	LM	HM
Shell length mm	26.3 ± 4.1	28.5 ± 3.7	29.6 ± 1.5	25.6 ± 4.0
Shell aperture mm	14.9 ± 1.2	15.5 ± 1.6	16.2 ± 0.7	14.8 ± 1.0
Total weight g	4.9 ± 1.7	5.6 ± 1.6	6.3 ± 0.6	4.5 ± 1.6
Soft body weight g	1.6 ± 0.5	1.9 ± 0.6	2.2 ± 0.5	1.4 ± 0.7

Reproduction

The cumulative number of neonates released per snail was highest in snails exposed to RW; snails exposed to RW released 14.7 neonates each, RW+S 13.2 neonates each, LM 12.4 and HM 13.6 each. However, there was no statistically significant difference between the four treatment groups (P = 0.451, ANOVA). Statistical analysis (ANOVA) at each sampling point found significant differences between the groups at weeks 8 (P = 0.002) and 10 (P = 0.042), but not at any other time (Figure 43). Post hoc analysis (LSD) found that at week 8 the snails exposed to RW released significantly more neonates, compared to snails exposed to RW+S (P = 0.002), LM (P = 0.001) and HM (P = 0.012). At week 10, the snails exposed to LM released significantly fewer neonates, compared to snails exposed to RW (P = 0.009) and RW+S (P = 0.048).

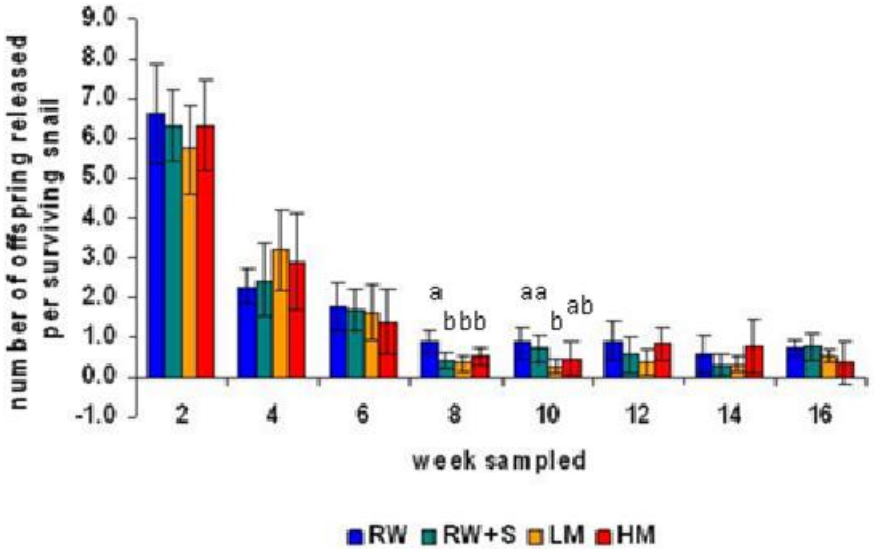

Figure 43. Mean number (± SD) of neonates released per adult *V. viviparus*, exposed to RW, RW+S, LM or HM over a 16-week period. Shared letters (a, b) indicate no statistically significant difference between these figures.

Number of embryos in brood pouch

Adult females from the RW treatment carried the highest mean total number of embryos at the end of the exposure (30.5 embryos per female). The lowest number of embryos per female (27.5) was from the HM treatment. There was no significant difference between the treatment groups (P = 0.630, ANOVA). The females exposed to RW carried the most hatched (mature) embryos and the least unhatched (immature) embryos. Conversely, females exposed to LM carried the fewest hatched embryos and the highest number of unhatched embryos (Table 59). There was no significant difference between treatments in the numbers of shelled (P = 0.128, ANOVA) or unshelled (P = 0.416, Kruskal-Wallis test) embryos carried per female, at the end of exposure.

Table 59. Mean number (± SD) of embryos per surviving adult female *V. viviparus*, following a 16-week exposure to RW, RW+S, LM or HM. RW, n = 33; RW+S, n = 27; LM, n = 35; HM, n = 28.

Embryos	RW	RW+S	LM	HM
Shelled	26.5 ± 11.1	23 ± 11.5	18.6 ± 9.7	23.25 ± 9.6
Un-Shelled	4.0 ± 3.5	5.7 ± 5.1	8.3 ± 6.8	4.25 ± 4.3
Total	30.5 ± 12.2	28.7 ± 12.5	26.9 ± 10.7	27.5 ± 9.8

Histopathology of adult *V.viviparus* exposed to estrogenic mixtures

Effects on oogenesis

Female *V. viviparus* exposed to RW had the highest mean number of oocytes per gonad section (11.0 oocytes per section). The females exposed to LM had approximately half as many oocytes per section (4.2 oocytes) (Figure 45). There was a

significant difference between the four exposure groups (P = 0.001, ANOVA). Post hoc analysis (LSD) found that RW females had significantly more oocytes per gonad section than snails exposed to LM (P < 0.001), HM (P = 0.001) and RW+S (P = 0.049).

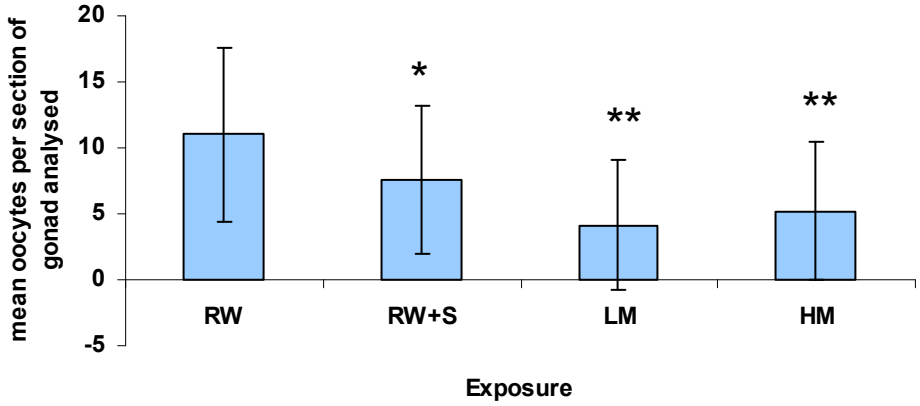

Figure 45. Mean number (± SD) of oocytes per gonad section from female *V. viviparus*. Measurements made after four months of exposure to RW, RW+S, LM or HM. A sample of females from each group were analysed histologically (RW, n = 23; RW+S, n = 22; LM, n = 21; HM, n = 21). Ten sections of gonad were analysed per snail. * indicates there is a significant difference from RW (P<0.05). ** indicates a significant difference from RW + S (P<0.005).

Effects on spermatogenesis

The mean spermatogenesis score was lowest in males exposed to RW+S. Mean scores from the other three treatments were similar (Table 62). There were no significant differences between the four treatment groups (P = 0.396 Kruskal-Wallis test).

Table 62. Mean (± SD) spermatogenesis score for adult male *V. viviparus*, after four months of exposure to RW, RW+S, LM or HM. All males were analysed histologically (RW, n= 4; RW+S, n= 6; LM, n= 5; HM, n =2). Ten sections of gonad were analysed per snail.

	RW	RW+S	LM	HM
Mean spermatogenesis score	10.0 ± 0.1	7.9 ± 3.6	9.9 ± 0.2	10.0 ± 0.0

Parasitism

Only four of the female snails examined had no parasite infection (2 in the RW and 2 in the RW+S treatments). Encysted parasites (metacercaria) in the muscular tissue of the head and the foot were most frequent, but infections of the digestive tissue (sporocyst stage) were also found.

The intensity of infection (parasite numbers or area infected) was recorded for both encysted and digestive tissue parasites. In adult female *V. viviparus*, the intensity of infection, by encysted parasites, was highest in snails exposed to HM and lowest in snails exposed to RW (Figure 46a). Only one male was found to be free of parasite infection. This individual was from the RW treatment. The majority of males had

encysted parasites (metacercaria) in the muscular tissue of the head and the foot, but one had an infection of the digestive tissues.
In adult male *V. viviparus*, the intensity of infection, by encysted parasites, was highest in snails exposed to LM and lowest in snails exposed to RW (Figure 46b).

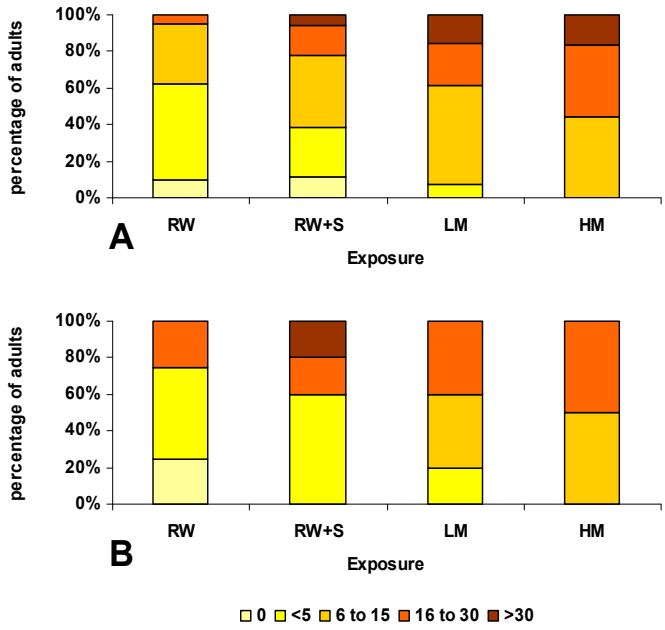

Figure 46. Histograms of encysted parasite infections in adult *V. viviparus*, after four months exposure to RW, RW+S, LM or HM. (A) Percentage of females with different intensities of parasite infection (RW, n = 23; RW+S, n = 22; LM, n = 21; HM, n = 21). (B) Percentage of males with different intensities of parasite infection (RW, n= 4; RW+S, n= 6; LM, n= 5; HM, n =2). Ten sections of tissue per adult snail were analysed. Pale yellow indicates no parasites found, yellow indicates less than 5 parasites per section, light orange indicates 5-15 parasites per section, dark orange indicates 15-30 parasites per section and brown indicates more than 30 parasites per section.

3.5.3 Discussion

The aim of this experiment was to explore the effects of a realistic mixture of oestrogenic chemicals, at concentrations expected to be found in sewage effluents or in rivers, on reproduction and development in gastropod snails. Also, to compare and contrast these effects with those seen in snails exposed to E2 (experiments 1 and 2) and whole effluents (Clarke et al. 2009). As was found in the E2 mesocosm studies, there was great variation in the measured concentrations of each chemical. The measured concentrations were generally much lower than the nominal concentrations, with the exception of EE2. The lower than expected concentrations of the alkylphenols and BPA, in both the dosed mesocosm tanks, is probably the result of using river water (which includes algae, bacteria, other biota and suspended sediments). Alkylphenols and BPA, in common with steroid oestrogens (experiment 2), are subject to sorbtion on to sediments (Ying et al. 2003), uptake by biota and bacterial degradation in the mesocosm tanks. The river water supplied to each tank included large volumes of suspended sediments; at the end of the 16 week exposure approximately 50 - 70mm of sediment had been deposited on the bottom of each tank. The added chemicals may also have been metabolised and/or degraded by bacteria in the river water (Kang and Kondo, 2002; Ike et al. 2000; Ahel et al. 1993; Ying et al. 2002, 2003; Langston et al.

2005; Johnson et al.1998) or bioaccumulated in the tissues of the snails and/or macro algae in the tank (Kang and Kondo, 2006; Takahashi et al. 2003). Ahel et al. (1993) reported extremely high bioaccumulation of NP, up to 10000 fold, in the filamentous blanket weed *Cladophora glomerata*. Filamentous blanket weed was present in all the mesocosm tanks and grew vigorously throughout the experiment. Microalgae (*Isochrysis galba*) have also been reported to bioaccumulate NP, up to 6940 times. The uptake was extremely fast, 77% of the NP added to the water had accumulated intracellularly after 1 hour (Correa-Reyes et al. 2007).

The concentrations of the chemicals in the dosing reservoirs were also variable. It is probable that both sorbtion and bacterial action affected the dosing reservoirs. A film (assumed to be bacterial) had formed on the surface of all the dosing reservoirs by the end of the 16 week experiment. There were also unexpectedly high concentrations of E2 and EE2 in the reference tanks and the river inlet. The reason for this is unknown. BPA was measured in the RW and the RW+S tanks, but BPA, NP and OP were not measured in the river water inlet, so the source of this BPA contamination is unknown. It is possible the BPA leached out of the plastics used in the mesocosm tank and/or pipes. The hot weather experienced during the experiment may have helped mobilise BPA from the plastic into the water.

In the RW+S tank there was a severe decline in the survival of the snails at week 10. As a consequence, only 14% of the *P. corneus* adults in this tank survived until the end of the 16 week study compared to 69% in all other treatments. The reason for this decline is unknown. Additional water chemistry samples were taken from the RW+S and RW tanks at week 16 and analysed for 19 metals, compounds and essential elements. The only difference between the tanks was a lower concentration of calcium in the RW tank (6.10mg/l), compared to the RW+S tank (94.1mg/l) and the RW tank used in the E2 study (96.8mg/l). It is possible that the carrier solvent (ethanol) affected snail survival. The concentration of solvent was not measured in the tanks, but an estimation of the amount entering the tanks can be made from the dosing pump rate and the volume of river water entering the tank. The dosing pump rate in the RW+S tank was, on average, higher than the HM or LM pump rates and the water flow rate into the tank was, on average, lower. Therefore, the concentration of ethanol in the RW+S tank was probably elevated, compared to its concentration in the LM and HM tanks. This increase in concentration was especially evident between weeks 10 and 12, which coincided with the beginning of the crash in adult snail survival in the RW+S tank. Hutchinson et al. (2006) reviewed the use of carrier solvents in eco-toxicology testing. The acute (48 hour) toxicity of ethanol was reported to be thousands of mg/l for a range of species, including the invertebrate *Daphnia magna* and the fish *Cyprinus carpio* (Hutchinson et al. 2006). These authors concluded that to avoid any adverse 'solvent effects' (including altered reproduction and enzymatic processes) solvent concentrations should be kept below 20μl/l. The maximum estimated ethanol concentration in the RW+S tank was 0.000654μl/l, compared to an estimated maximum of 0.000583μl/l in the mixture tanks. These concentrations are orders of magnitude below the solvent concentrations reported to cause effects on reproduction. Indeed, they were so low as to be biologically insignificant. While the interpretation of this experiment is necessarily limited, the results do provide some very interesting preliminary evidence of the effects of mixtures of chemicals on gastropod snails.

Despite the increased mortality in the RW+S tank, there were no obvious consistent differences in the number of juvenile snails or eggs released by adults, of either species, over the entire 16 week experiment. The *V.*viviparus snails in the RW tank produced significantly more neonates at week 8 and histological analysis of the females, after 16 weeks of treatment, found the snails exposed to RW had significantly more oocytes than the females exposed to LM and HM. Taken together this indicates a level of disruption to oogenesis, rather than stimulation. Exposure of adult brown

trout (*Salmo trutta*) to BPA reportedly resulted in a dose-dependent delay in ovulation at concentrations of 1.75 and 2.40μg/l, and no ovulation at all at 5μg/l (Lahnsteiner et al. 2005). Further experiments could establish the existence of any dose response relationship.

There were no significant differences in the reproductive output of adult *P. corneus* between the different treatments. However, over the whole experiment, the snails exposed to LM laid both the most egg masses and the largest egg masses. Similar results were reported when *P. corneus* was exposed to TSE (Clarke et al. 2009). The mechanism underlying this effect is not known. Mice exposed to BPA have been reported to up-regulate the dopamine D1 receptor function (Suziki et al. 2003). This effect may be relevant in freshwater gastropods as dopamine has a stimulatory effect on protein secretion in the albumen gland (Santhanagopalan and Yoshino, 2000) and this activity is mediated via a D1-like receptor (Mukai et al. 2004). If EDCs disrupt receptor function in molluscs, this could affect the volume of albumen deposited around each egg.

Histological analysis of the gonads of adult *P. corneus* found some effects of exposure on cells supporting gametes. The Sertoli cells seemed to be especially sensitive. In snails exposed to LM and HM, there were significant increases in the amount of sloughing of Sertoli cells into the acini lumen, compared to snails exposed to RW. Sloughing of spermatogonia and spermatids was most frequent in snails exposed to LM. The frequency of spermatocyte sloughing was significantly higher in snails exposed to HM, and was also elevated in snails exposed to LM, compared to snails in the RW treatment. In snails exposed to HM there was a significant reduction in the number of developing sex cells (male and female) that were covering the acini walls, compared to the RW and RW+S treatments. As previously discussed, E2 has been reported to have negative effects on Sertoli cell function in vertebrates (Miles-Richardson et al. 1999; Gill-Sharma et al. 2001; Rasmussen et al. 2005; Aleem et al. 2006). BPA, NP and OP also affect Sertoli cell structure and function. There was a reduction in the viability of rat Sertoli cells, cultured *in vitro*, when they were exposed to BPA (150-200μM) or OP (30-60μM) (Iida et al. 2003; Qian et al. 2006). Exposing Eelpout (*Zoarces viviparus*) to OP (50 and 100μg/l) for three weeks, resulted in alterations in Sertoli cell structure (Rasmussen, 2005). In Fathead minnows exposed to NP (1.1 and 3.4μg/l), there were changes in the number and size of Sertoli cells, but these concentrations did not affect external sexual characteristics (e.g. fat pad). It is possible that similar effects are occurring in this gastropod model.

2.6 Mesocosm experiment 3b: An analysis of the morphology and histology of the F1 generation developmentally exposed to estrogenic mixtures

2.6.1 Methods

At the end of the adult exposure (September) all the F1 snails were collected and measured. Fifty *V. viviparus* and 40 *P. corneus* (only 20 *P.corneus* from the HM tank) that were over 10mm in size were sacrificed for histological analysis. The mesocosm tanks were re-filled with river water and the remainder of the F1 snails were allowed to depurate over winter. The following April a breeding study was set up. This followed the protocol used in Experiment 2b, except that, in the *P.corneus* experiment, only one replicate of four *P.corneus* was deployed in the HM tank.

2.6.2 Results

Effects of exposure to estrogenic mixtures on F1 *P.corneus* survival, growth and development.

Survival

At the end of the exposure period, the largest number of F1 *P. corneus* were found in the LM and HM tanks (RW = 239, RW+S = 213, LM = 2129, HM = 461). However, the vast majority of F1s collected from the LM tank were very small (2000 young hatchlings). The highest percentages of F1 snails above 10mm shell diameter (normal minimum size for sexually active snails) were in the RW (44%) and RW+S tanks (39%). The percentages of F1s, above 10mm diameter, in the mixture tanks were low (HM, 5% and LM, 6%).

Growth

A sample of 20 F1s from each treatment were analysed histologically. The F1s that were exposed to HM during their development were significantly smaller (shell diameter) and lighter (total weight and soft body weight) than any of the other treatment groups (Figure 47).

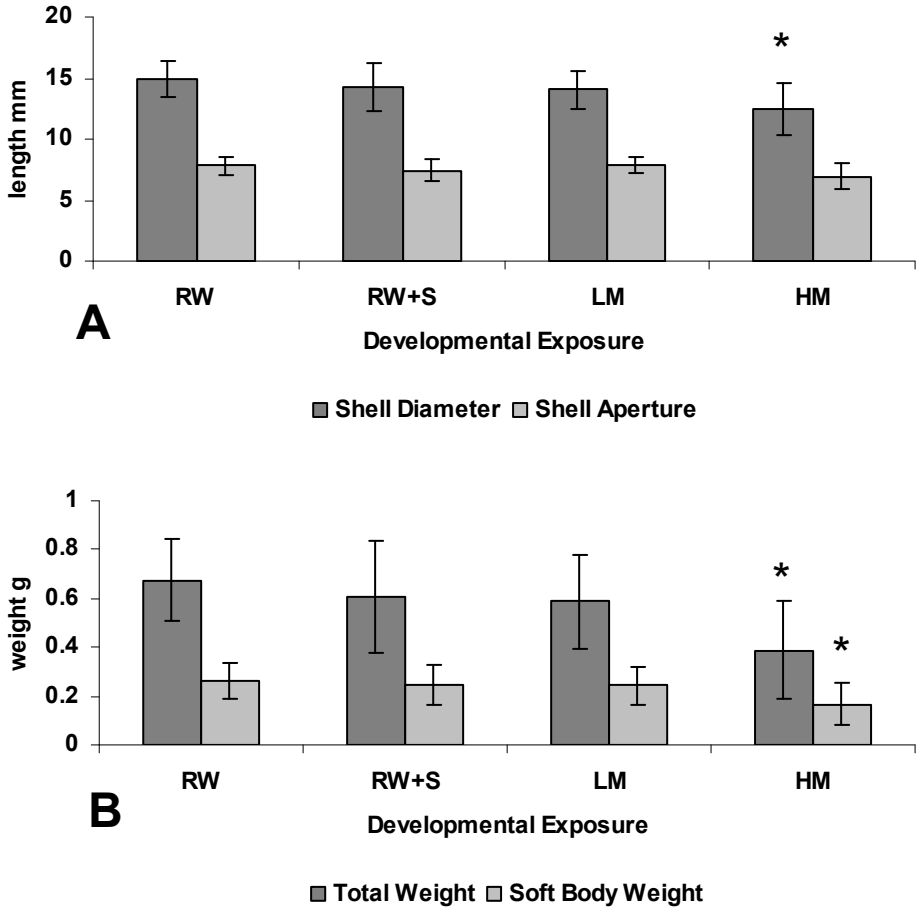

Figure 47. (A) Mean (± SD) shell length and aperture and (B) total weight and soft body weight of F1 *P. corneus*. *S*amples were taken from the four treatment tanks in September 2006. * indicates a statistically significant difference (P <0.05) from other treatments (ANOVA).

Histopathology of *P. corneus* F1 gonad immediately after exposure to estrogenic mixtures

In September 2006 (end of exposure), approximately 40 F1s (only 20 from the HM treatment) that were above 10mm shell diameter (presumed sexually mature) were removed from each treatment for histological analysis. Due to the complex nature of the *P. corneus* gonad, the analysis was split between different areas, or cell types, within the gonad. The majority of snails sampled, from all the treatments, had all stages of oogenesis and spermatogenesis present in the gonad.

Effects on Acini

Three snails, one each from the HM, RW+S and RW treatments, had no Sertoli cells attached to the acini walls in one out of five gonad sections. One snail from the RW+S treatment had no Sertoli cells attached to the acini walls in two out of five gonad sections. All other snails (from all four treatments) had Sertoli cells attached to the acini walls in five out of five gonad sections. Analysis, using the Kruskal-Wallis test, found no significant difference between the four treatment groups.

Table 67. The percentage of F1 *P. corneus* snails with different levels of activity in vitellogenic area of the acini. Samples were taken after developmental exposure to RW, RW+S, LM or HM. Histological analysis was performed on a sample of 20 F1s from each treatment. Five gonad sections were analysed per F1 snail. Shared letters (a and b) denote no statistically significant difference between groups.

% of acini with active vitellogenic area	Score	% of F1 snails			
		RW	RW+S	LM	HM
<10%	1	10%	0	0	6%
10-30%	2	10%	5%	5%	24%
30-50%	3	0	30%	37%	29%
50-70%	4	46%	50%	47%	41%
>70%	5	35%	15%	11%	0
		a	A	a	b

There was a dose-dependent decrease in normal activity in the vitellogenic area (maturing oocytes with follicle cells and/or sperm with Sertoli cells) (Table 67). A Oneway ANOVA test found there was a significant difference between the four exposure groups (P = 0.015). Post hoc analysis (LSD) found that F1s exposed to HM had significantly less active vitellogenic areas, compared to all other treatment groups. F1s exposed to LM during development had the least disruption to the acini wall, while those from the RW+S treatment had the most (Table 68). A Kruskal-Wallis test found no significant difference between the four different treatment groups.

Table 68. Percentage of F1 *P. corneus* snails with different levels of disruption to the acini wall. Samples taken after exposure to RW, RW+S, LM or HM during development. Histological analysis was performed on a sample of 20 F1s from each treatment. Five gonad sections were analysed per F1 snail.

	% of F1 *P. corneus* snails with acini wall disruption			
Number of sections affected	**RW**	**RW+S**	**LM**	**HM**
0 out of 5	55%	35%	68%	65%
1 out of 5	0	10%	16%	5%
2 out of 5	0	15%	0	10%
3 out of 5	20%	15%	5%	10%
4 out of 5	10%	15%	0	10%
5 out of 5	15%	10%	11%	0

The cell coverage (developing oocytes, sperm and supporting cells) on the acini walls was highest in F1 *P. corneus* exposed to LM and lowest in snails exposed to RW+S (Table 69). Analysis, using a Kruskal-Wallis test, found there was a significant difference between treatment groups (P <0.001). Post hoc analysis (Mann-Whitney test) found there were no significant differences between snails exposed to HM and LM, or between snails exposed to RW and RW+S. However, snails exposed to RW and RW+S had significantly less cell coverage on their acini walls than snails from the HM and LM treatments.

Table 69. The percentage of F1 *P. corneus* snails with different levels of cell coverage on their acini walls, after developmental exposure to RW, RW+S, LM or HM. Histological analysis was performed on a sample of 20 F1s from each treatment. Five gonad sections were analysed per F1 snail. Shared letters (a, b) indicate no statistically significant difference between these groups.

Percent of acini wall covered by germ cells and/or supportive cells	Score	% of F1 *P. corneus* snails			
		RW	RW+S	LM	HM
<10%	1	10%	25%	0	5%
10-30%	2	45%	55%	5%	30%
30-50%	3	30%	20%	30%	10%
50-70%	4	5%	0	35%	25%
>70%	5	10%	0	30%	30%
		a	a	b	b

Effects on spermatogenesis

Sertoli cells sloughing into lumen

F1s from the RW+S treatment had the least number of gonad sections where Sertoli cells were sloughing into the acini lumen, snails exposed to LM had the most (Table 70). An ANOVA test found there was no significant difference between the four treatment groups.

Spermatogonium sloughing into lumen

F1s from the LM treatment had the least number of gonad sections where spermatogonia were sloughing into the acini lumen, snails exposed to HM had the most (Table 70). An ANOVA test found there was no significant difference between the four treatment groups.

Spermatocyte sloughing into the lumen

F1s from the RW+S treatment had the least number of gonad sections where spermatocytes were sloughing into the acini lumen, snails exposed to RW had the most (Table 70). A Kruskal-Wallis test found there was no significant difference between the four treatment groups.

Spermatids sloughing into the lumen

F1s from the RW treatment had the least number of gonad sections where spermatids were sloughing into the acini lumen, snails exposed to HM had the most (Table 70). A Oneway ANOVA test found there was a significant difference between treatment groups ($P = 0.002$). Post hoc analysis found that snails exposed to RW and RW+S had significantly less sloughing of spermatids in to the lumen than snails exposed to LM and HM. There was no significant difference between snails treated with RW and RW+S snails or between snails treated with LM and HM.

Table 70. The percentage of F1 *P. corneus* snails with different levels of sloughing of immature spermatogenic cells into the acini lumen. Snails examined after developmental exposure to RW, RW+S, LM or HM. Histological analysis was performed on a sample of 20 F1s from each treatment. Five gonad sections were analysed per F1 snail. Shared letters (a, b) indicate no statistically significant difference between those groups.

Number of sections affected	Percentage of F1s affected by Sertoli cell sloughed into lumen				Percentage of F1s affected by spermatogonia sloughed into lumen				Percentage of F1s affected by spermatocytes sloughed into lumen				Percentage of F1s affected by spermatids sloughed into lumen			
	RW	RW+S	LM	HM	RW	RW+S	LM	HM	RW	RW+S	LM	HM	RW	RW+S	LM	HM
None	65	60	50	55	10	5	0	0	45	65	50	40	55	35	10	5
1 out of 5	0	15	10	10	5	0	10	0	20	30	5	10	0	15	10	10
2 out of 5	10	20	15	10	0	0	10	0	5	0	15	10	10	15	10	15
3 out of 5	15	0	10	15	10	5	5	15	0	0	5	25	10	15	15	20
4 out of 5	10	0	5	5	10	20	30	15	5	0	10	0	15	5	20	10
5 out of 5	0	5	10	5	65	70	45	70	25	5	15	15	10	15	35	40
	a	a	a	a	a	a	a	A	A	a	a	a	A	a	b	b

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Spermatozoa

F1s that had been exposed to RW had the highest number of gonad sections where only spermatozoa were free in the acini lumen, snails exposed to HM had the fewest gonad sections containing only free spermatazoa (Table 71). There was no significant difference between the treatment groups.

Table 71. The percentage of F1 *P. corneus* snails with different numbers of gonad sections with only mature spermatozoa in the acini lumen, after developmental exposure to RW, RW+S, LM or HM. Histological analysis was performed on a sample of 20 F1s from each treatment. Five sections of gonad were analysed per F1 snail.

No. of sections	% of F1 *P. corneus* snails with mature spermatozoa in the acini lumen			
	RW	RW+S	LM	HM
0 out of 5	70	70	55	85
1 out of 5	5	25	35	10
2 out of 5	10	0	5	5
3 out of 5	0	0	0	0
4 out of 5	5	0	5	0
5 out of 5	10	5	0	0

The percentage of acini (per section of gonad) where immature spermatogenic cells were sloughing into the lumen, was highest in snails exposed to RW and lowest in

snails exposed to RW+S. An ANOVA test found no significant difference between the four treatment groups.

Effects on oogenesis

Similar patterns of oocyte development were seen among F1s exposed to RW and RW+S - similar percentages of each oogenesis stage were present in the gonads of these snails (Table 73). Snails exposed to LM during development had the highest percentage of young oocytes (stage 1 and 2) and the highest percentage of mature oocytes (stage 5). Statistical analysis (ANOVA) found there were no significant differences in the percentage of stage 1 and 2 oocytes between the four treatments. Post hoc analysis (LSD) found snails exposed to RW and RW+S had a significantly lower percentage of stage 1 oocytes, than snails from the LM and HM treatments. There was no significant difference in the percentage of stage 1 oocytes between snails exposed to RW+S and RW, or between snails exposed to LM and HM. Post hoc analysis found snails exposed to LM during development had a significantly higher percentage of stage 2 oocytes, compared to snails exposed to HM. There was also a significant difference in the percentage of degenerating oocytes ($P > 0.001$, Kruskal-Wallis test). Post hoc analysis (Mann-Whitney test) found snails exposed to HM had a significantly lower percentage of degenerating oocytes than snails exposed to RW and RW+S. Snails exposed to RW+S had a significantly higher percentage of degenerating oocytes than snails exposed to LM.

Table 73. Mean percentage of different stages of oogenesis in F1 *P. corneus*, after developmental exposure to RW, RW+S, LM or HM. Histological analysis was performed on a sample of 20 F1s from each treatment. Five gonad sections were analysed per F1 snail. Stage 1 (oogonium) is the earliest stage of oogenesis, stage 5 is ready to be ovulated. Oogenesis stages are based on the descriptions by de Jong-Brink et al. (1979). Shared letters (a, b) indicate there is no statistically significant difference between these figures.

Stage of oocyte maturation	Mean % of F1 snails at each oogenesis stage			
	RW	RW+S	LM	HM
Stage 1	10.7 ± 7.9 a	10.7 ± 9.8 a	16.5 ± 7.7 b	18.3 ± 5.2 b
Stage 2	15.8 ± 4.9 ab	14.9 ± 7.0 ab	18.0 ± 5.0 a	13.0 ± 4.6 b
Stage 3	24.3 ± 6.5	20.3 ± 6.6	22.2 ± 5.2	12.6 ± 6.5
Stage 4	23.9 ± 11.4	23.7 ± 13.4	22.2 ± 7.2	29.5 ± 9.3
Stage 5	10.1 ± 6.4	10.7 ± 9.8	12.8 ± 6.9	11.6 ± 6.3
Degenerating	15.3 ± 12.1 a	19.7 ± 12.6 a	8.3 ± 7.4 ab	5.9 ± 7.9 b

Parasitism

None of the F1 *P. corneus* had any parasitic infection.

Effects of exposure to estrogenic mixtures on F1 *P. corneus* survival, growth and reproduction following depuration.

Survival

The over winter survival of F1 *P. corneus* was highest in snails exposed to RW during development (60 snails = 19%), compared to much lower survival rates among snails from the LM (123 snails = 6%) and HM (8 snails = 2%) treatments. Between March and April 2007 there were similar percentages of surviving snails from the RW (48 snails = 80%) and LM (105 snails = 85.4%) treatments. The snails exposed to HM during development continued to have a lower percentage survival (5 snails = 62.5%).

Over the 14 weeks of the breeding study, snails exposed to RW suffered the highest percentage mortality (8 snails = 44% surviving). By the end of the study, survival among snails exposed to HM and LM was 50% (2 snails) and 88.9% (16 snails), respectively. Survival was significantly higher in snails exposed to LM, than snails exposed to RW, at weeks 2 to 14 (inclusive).

Growth

The F1 *P. corneus s*nails were measured in April 2007, after eight months of depuration. The snails exposed to the mixtures were on average smaller than those from the RW treatment. The snails from RW had a mean shell diameter of 14.6 $\pm$ 1.9mm, compared to 13.9 $\pm$ 0.6mm (LM snails) and 11.8 $\pm$ 1.6mm (HM snails) (Table 77). There were significant differences between the groups for shell length (P =0.018,), shell aperture (P = 0.015) but not for total weight (P = 0.059) (Kruskal-Wallis test). Post hoc analysis found that RW and LM snails were significantly larger than HM snails for shell diameter, shell aperture and weight. There were no significant differences between the snails from the RW and LM treatments.

Table 77. Shell diameter and total weight of F1 *P. corneus* at the start and end of the 14 week un-dosed F1 breeding study. Snails had previously been exposed to RW, LM or HM during their development and then depurated in river water for eight months.

	RW	LM	HM
Start of study Shell diameter mm	14.6 $\pm$ 1.9	13.9 $\pm$ 0.9	11.8 $\pm$ 1.6
End of study Shell diameter mm	16.3 $\pm$ 1.6	15.8 $\pm$ 1.3	15.8 $\pm$ 2.1
Start of study Total weight g	0.7 $\pm$ 0.3	0.6 $\pm$ 0.1	0.35 $\pm$ 0.2
End of study Total weight g	0.9 $\pm$ 0.23	0.8 $\pm$ 0.2	0.8 $\pm$ 0.3

At the end of the 14 week reproductive study, the snails exposed to RW during their development were still larger than the snails that had been exposed to LM or HM. However, statistical analysis (ANOVA) found no significant differences between the treatment groups in shell diameter (P = 0.725), shell aperture (P = 0.162), total weight (P = 0.667) or soft body weight (P = 0.654). Over the breeding study, growth was highest in the HM group with a 25.5% increase in shell diameter and 56.6% increase in total weight. Growth in shell length of LM and RW snails was 12.3% and 10.8%, respectively. The increases in total weight for LM and RW snails were 27.5% and 25.9%, respectively.

Reproduction

Reproductive output of *P. corneus*, exposed to HM during development, was severely affected in comparison to snails exposed to RW or LM (Figure 48). Statistical analysis (t-test) found that snails exposed to RW laid significantly more eggs per snail at week 2 than snails exposed to LM. However, at the end of the breeding experiment there was no significant difference between snails exposed to RW and LM, in terms of the cumulative number of eggs laid per snail. Egg laying in the HM treatment could not be analysed as there was only one replicate group, but there was a clear effect on egg laying; the total number of eggs laid per snail did not exceed 1, at any sampling point.

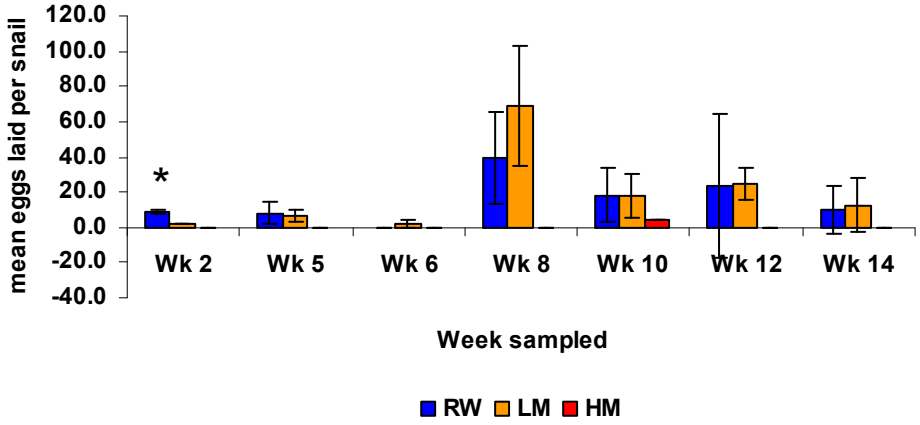


Figure 48. Mean (± SE) of eggs laid during the breeding study, by F1 adult *P. corneus*. The snails had previously been exposed to RW, LM or HM during development and then depurated over autumn/winter in river water. * indicates a statistically significant difference from LM.

Histopathology of F1 *P. corneus* gonads following depuration

Histological analysis was performed on all surviving snails from the F1 breeding study (RW, n = 8; LM, n = 16; HM, n = 2). Five gonad sections were analysed per snail.

Effects on Acini

All of the gonad sections, from all snails, from all three treatments, had Sertoli cells attached to the acini walls. A dose-dependent decrease in normal activity in the vitellogenic area (maturing oocytes with follicle cells and/or sperm with Sertoli cells) was found in snails that had been exposed to LM or HM during development and then depurated in river water for approximately one year (Table 78). ANOVA found there was no significant difference between the three groups. The majority of F1 snails (from all treatments) had gonad sections with intact acini walls. ANOVA found no significant difference between the exposure groups.

Table 78. The percentage of F1 *P. corneus* snails with different levels of activity in the vitellogenic area of the acini. Snails were examined after exposure to RW, LM or HM during their development followed by depuration for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW, n = 8; LM, n = 16; HM, n = 2). Five gonad sections were analysed per snail.

Percent of acini with active vitellogenic area	Score	Percentage of depurated F1 snails from the river water exposure (RW)	Percentage of depurated F1 snails from the low mixture exposure (LM)	Percentage of depurated F1 snails from the high mixture exposure (HM)
<10%	1	0	0	0
10-30%	2	0	12%	50%
30-50%	3	13%	47%	50%
50-70%	4	49%	35%	0
>70%	5	38%	6%	0

Depurated snails that had been exposed to HM during development had the most cell coverage (developing oocytes, sperm and supporting cells) on the acini walls. However, only two HM snails were examined due to the high mortality in this group. Snails that had been exposed to RW had more cell coverage than snails exposed to LM (Table 79). A Kruskal-Wallis test found there was no significant difference between the treatment groups.

Table 79. Percentage of depurated F1 *P. corneus* snails with different levels of cell coverage on the acini wall. Snails had been exposed to RW, LM or HM during their development and then depurated for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW n = 8; LM, n = 16; HM, n = 2). Five gonad sections were analysed per snail.

Percent of acini wall covered by germ cells and/or supportive cells	Score	Percentage of depurated F1 snails from the river water exposure (RW)	Percentage of depurated F1 snails from the low mixture exposure (LM)	Percentage of depurated F1 snails from the high mixture exposure (HM)
<10%	1	0	0	0
10-30%	2	0	12.5	0
30-50%	3	13	31.25	0
50-70%	4	25	12.5	0
>70%	5	62	43.75	100

Effects on spermatogenesis

Sertoli cells sloughing into lumen

In snails exposed to LM and RW, similar numbers of gonad sections were found with Sertoli cells sloughed into the acini lumen (Table 80). Snails treated with HM had the lowest percentage of gonad sections where Sertoli cells were sloughing into the lumen. ANOVA found there was no significant difference between the treatment groups.

Spermatogonia sloughing into lumen

Sloughing of spermatogonia into the acini lumen was seen most frequently in snails that had been exposed to HM during development (Table 80). ANOVA found there was no significant difference between the treatment groups.

Spermatocyte sloughing into the lumen

Sloughing of spermatocytes into the lumen was seen most frequently in snails that had been exposed to HM during development. The lowest frequency of sloughing was in snails exposed to RW (Table 80). There was no significant difference between the treatment groups (P = 0.134, Kruskal-Wallis test).

Spermatids sloughing into the lumen

Sloughing of spermatids into the lumen was seen most frequently in snails that had been exposed to RW during development. Snails exposed to LM showed a similar trend (Table 80). ANOVA found there was no significant difference between the treatment groups.

Table 80. The percentage of depurated F1 *P. corneus* snails with different levels of immature spermatogenic cells sloughed into the acini lumen. Snails had been exposed to RW, LM or HM during their development and then depurated for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW, n = 8; LM, n = 16; HM, n = 2). Five gonad sections were analysed per snail.

Number of sections affected	% of depurated F1s with spermatogenic cells sloughing into lumen											
	Sertoli Cells			Spermatogonia			Spermatocytes			Spermatids		
	RW	LM	HM	RW	LM	HM	RW	LM	HM	RW	LM	HM
None	37.5	37.5	0	25	12.5	0	37.5	37.5	0	25	12.5	0
1 out of 5	12.5	18.75	50	0	0	0	12.5	6.25	0	0	25	0
2 out of 5	12.5	6.25	0	12.5	18.75	0	0	18.75	0	12.5	0	50
3 out of 5	0	6.25	50	0	18.75	0	12.5	6.25	0	12.5	12.5	0
4 out of 5	25	18.75	0	25	18.75	0	37.5	6.25	0	12.5	18.75	50
5 out of 5	12.5	12.5	0	37.5	31.25	100	0	25	100	37.5	31.25	0

Spermatozoa

The percentage of snails where only mature spermatozoa were free in the acini lumen was highest in snails exposed to RW during development. It decreased in a dose-dependent manner in the LM and HM treatments (Table 81). ANOVA found there was no significant difference between the treatment groups.

Table 81. The percentage of depurated F1 *P. corneus* snails with different numbers of gonad sections with only mature spermatozoa in the acini lumen. Snails had been exposed to RW, LM or HM during their development then depurated for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW, n = 8; LM, n = 16; HM, n = 2). Five gonad sections were analysed per snail.

	% of F1 *P. corneus* snails with only mature spermatozoa in the lumen		
Number of sections	**RW**	**LM**	**HM**
0 out of 5	50	56.25	100
1 out of 5	25	12.5	0
2 out of 5	0	6.25	0
3 out of 5	0	12.5	0
4 out of 5	0	6.25	0
5 out of 5	25	6.25	0

Snails that had been exposed to LM and then been depurated, had the highest percentage of snails with the most (>70%) acini where immature spermatogenic cells were sloughed into the lumen. No trend was apparent between treatments and ANOVA found there was no significant difference between the treatment groups.

Effects on oogenesis

Snails exposed to HM during development had the highest percentage of young oocytes (stage 1 and 2). The snails exposed to RW had the highest percentage of mature (stage 5) oocytes (Table 83). There were no significant differences between the treatment groups for any of the oocyte stages.

Table 83. Mean percentage (± SD) of different stages of oogenesis in F1 *P. corneus*. Snails had been exposed to RW, LM or HM during their development then depurated for approximately one year in river water. Histological analysis was performed on all snails from the F1 breeding study (RW, n = 8; LM, n = 16; HM, n = 2). Five gonad sections were analysed per snail. Stage 1 (oogonium) are the earliest stage of oogenesis, stage 5 is ready to be ovulated. The oogenesis stages were based on the descriptions by de Jong-Brink et al. (1979).

Stage of oocyte maturation	**Mean percent of oogenesis stage after river water exposure**	**Mean percent of oogenesis stage after LM exposure**	**Mean percent of oogenesis stage after HM exposure**
Stage 1	19.9 ± 5.5	22.7 ± 7.5	34.2 ± 11.2
Stage 2	21.8 ± 4.7	20.4 ± 3.5	16.8 ± 0.1
Stage 3	20.0 ± 4.2	19.6 ± 6.5	16.0 ± 2.2
Stage 4	19.9 ± 6.5	15.8 ± 5.3	24.9 ± 5.4
Stage 5	3.6 ± 3.3	1.9 ± 2.4	3.1 ± 1.9
Degenerating	14.8 ± 8.3	19.6 ± 10.1	5.1 ± 6.0

Parasitism

No parasites were found in any of the F1 snails from any of the exposure groups.

Effects of exposure to estrogenic mixtures on F1 *V. viviparus* survival, growth and development.

Survival

The number of neonates collected from the mesocosm tanks, at the end of the 16 week dosing period, was highest in the RW tank (553) and lowest in the RW+S tank (100). The LM tank contained 250 neonates and the HM tank 140. The percentage of snails with shells over 10mm long (minimum size of sexually active snails) was highest in the RW tank (80%). In the RW+S tank 49%, in the LM tank 60% and in the HM tank 64%, of F1s had shells over 10mm in length.

Growth

Females exposed to RW during development were the largest, while those exposed to HM were smallest. A Oneway ANOVA found there was a significant difference, in shell length, between treatment groups ($P = 0.004$). Post hoc analysis (LSD) found females from the RW treatment had significantly longer shells, compared to females from the HM or RW+S treatments. Females from HM treatment were also significantly smaller than females exposed to LM. The total weight of the females was analysed using the Kruskal-Wallis test. There was a significant difference between the four treatment groups ($P = 0.012$). Post hoc analysis found that the total weight of F1 females exposed to RW, was significantly greater than HM and RW+S females, but was not significantly different from that of snails exposed to LM. There was a significant difference between the four treatment groups for soft body weight (shell removed) ($P = 0.039$, ANOVA). Post hoc analysis (LSD) found that only RW and HM treatments ($P = 0.009$) were significantly different from one another (RW heavier than HM, Figure 51).

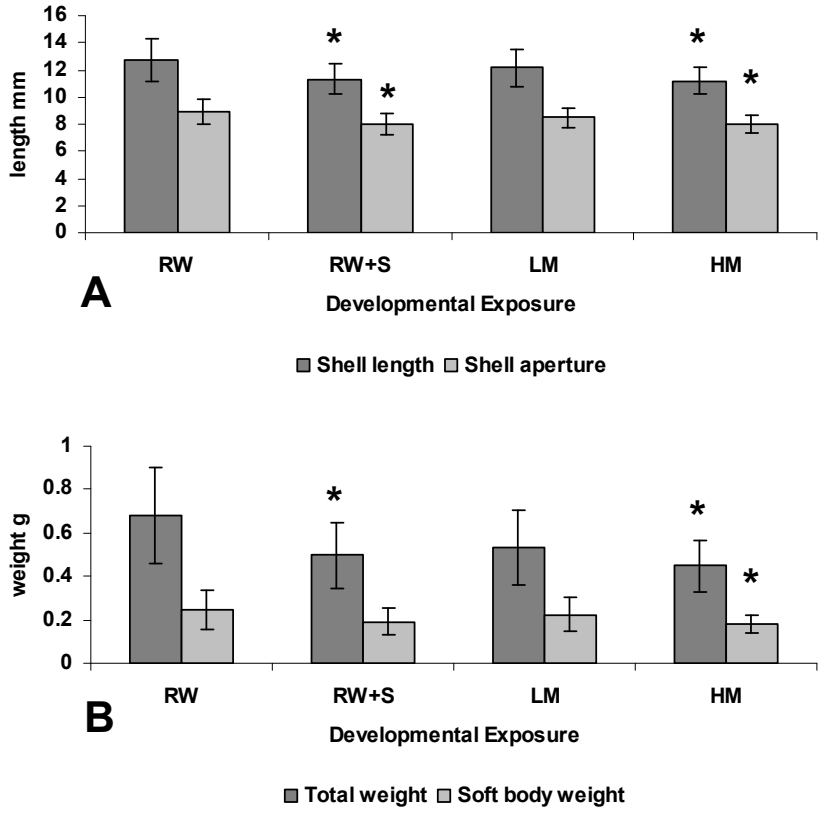


Figure 51. F1 *V. viviparus* females sampled in September 2006, after exposure to RW, RW+S, LM or HM during their development. RW, n = 15; RW+S, n = 13; LM, n = 20; HM, n = 18. (A) Mean shell length and shell aperture, (B) Mean total weight and soft body weight. Error bars show standard deviation. * indicates significant difference (P<0.05) from RW.

F1 males that had been exposed to RW were largest and males exposed to HM were smallest (Figure 52). Analysis of shell length, by Oneway ANOVA, found there was a significant difference between treatment groups (P = 0.003). Post hoc analysis (LSD) found that snails exposed to HM were significantly smaller than snails exposed to RW, RW+S and LM. There was a significant difference, between treatment groups, for total weight (P = 0.006, ANOVA). Post hoc analysis (LSD) found that snails exposed to HM were significantly lighter than snails exposed to RW, RW+S and LM. There was a significant difference, between treatments, in soft body weight (P = 0.017, ANOVA). Post hoc analysis (LSD) found that snails exposed to HM were significantly lighter than snails exposed to RW, RW+S and LM.

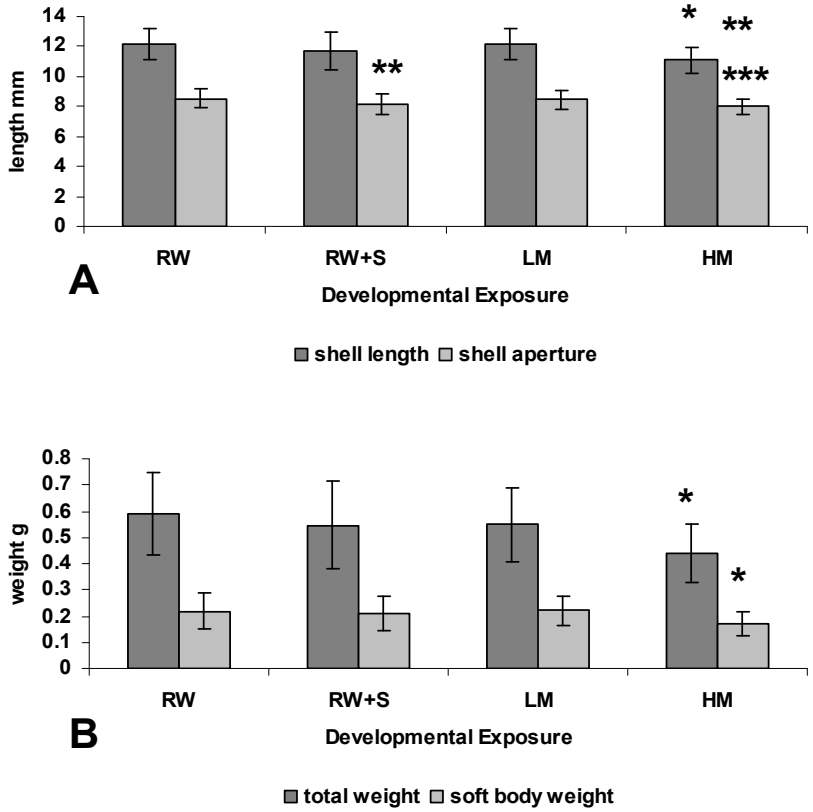


Figure 52. F1 *V. viviparus* males sampled in September 2006 after they had been exposed to RW, RW+S, LM or HM during their development. RW, n = 21; RW+S, n = 26; LM, n = 21; HM, n = 24. (A) Mean (± SD) shell length and shell aperture. (B) Mean (± SD) total weight and soft body weight. * indicates a significant difference (P <0.05) from all other treatment groups. ** indicates a significant difference from the RW group. * indicates a significant difference from the LM group.**

Histopathology of *V. viviparus* F1 gonads after developmental exposure to estrogenic mixtures

In September 2006 (end of exposure) approximately 40 F1s, with shells over 10mm long (presumed sexually mature), were removed from each treatment for analysis. Twenty one males and 15 females were taken from the RW treatment, 26 males and 13 females from the RW+S treatment, 21 males and 20 females from the LM treatment and 24 males and 18 females from the HM treatment.

Effects on oogenesis

The mean number of oocytes per gonad section was highest in F1s that had been exposed to RW and lowest in those exposed to HM. The females that had been exposed to RW during development had an average of 3.8 $\pm$ 4.5 oocytes per gonad section. Those exposed to RW+S had 2.6 $\pm$ 2.3 oocytes per section and those exposed to LM and HM had 3.1 $\pm$ 1.8 and 1.6 $\pm$ 1.6 oocytes per section, respectively (Figure 53). Analysis, using a Kruskal-Wallis test, found there was a significant difference between the treatment groups (P = 0.023). Post hoc analysis (Mann-Whitney test) found that the females exposed to HM had significantly fewer oocytes per section, compared to snails exposed to RW and LM.

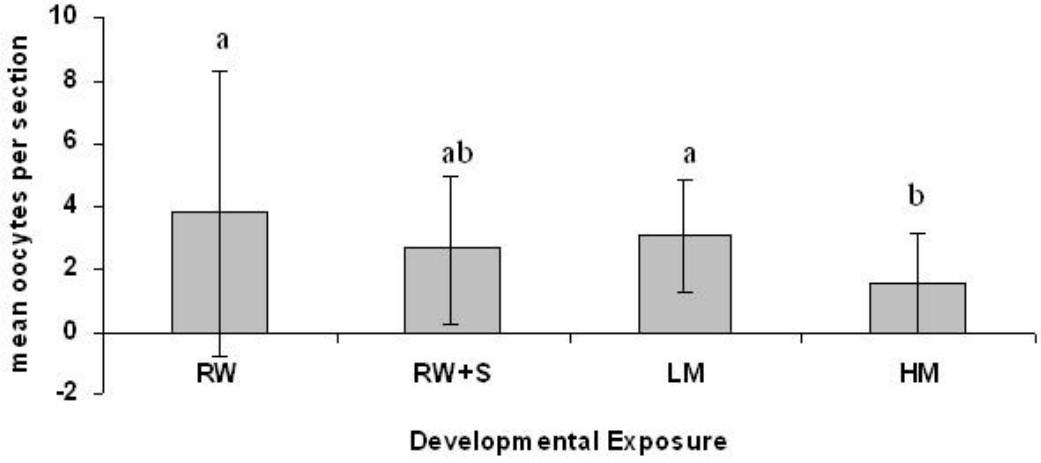


Figure 53. Mean (± SD) number of oocytes per gonad section from F1 *V. viviparus* females. Snails were sampled in September 2006 after developmental exposure to RW, RW+S, LM or HM. RW, n = 15; RW+S, n = 13; LM, n = 20; HM, n = 18. Five gonad sections were analysed per female. Shared letters (a, b) indicate there is no statistically significant difference between treatments.

Effects on spermtogenesis

Mean spermatogenesis scores were highest in snails that had been exposed to RW+S and lowest in snails exposed to HM (Figure 54). Analysis, by Kruskal-Wallis test, found there was a significant difference between the four treatment groups (P = 0.005). Post hoc analysis (Mann-Whitney test) found that males that had been exposed to HM had a significantly lower spermatogenesis score than snails exposed to RW (P = 0.011), RW+S (P = 0.002) or LM (P = 0.007). There were no significant differences between the RW and RW+S (P = 0.324) treatments, the RW and LM (P = 1.00) treatments, or between the RW+S and LM (P = 0.372) treatments.

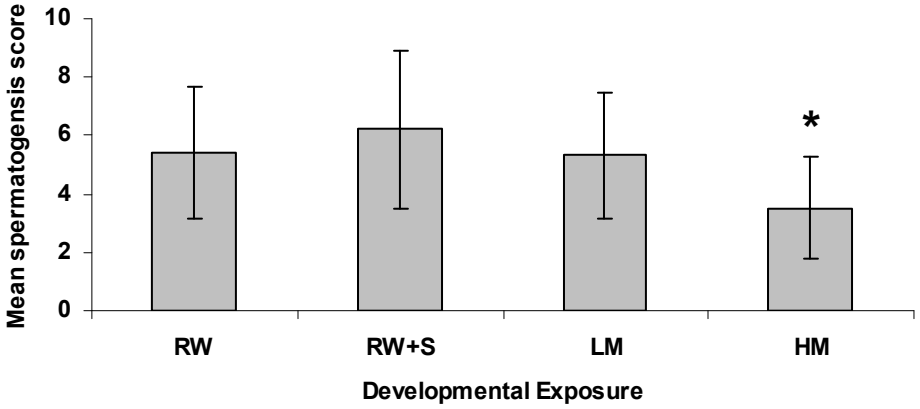


Figure 54. Mean (± SD) spermatogenesis score in F1 *V. viviparus* males. Snails were sampled in September 2006, after exposure to RW, RW+S, LM or HM during their development. RW, n = 21; RW+S, n = 26; LM, n = 21; HM, n = 24. Score 1 - no germ cells present, score 10 - full spermatogenesis in more than 70% of tubules. Five gonad sections were analysed per male. * indicates a significant difference (P <0.05) from all other treatment groups.

Parasitism

In the majority of both male and female F1 snails exposed to LM or HM, encysted parasites (metacercaria) were found in the muscular tissue of the head and the foot (Table 85). In snails exposed to RW or RW+S, the frequency of parasitism was low. No parasitic infections of the digestive tissue were seen in any of the F1s analysed.

Table 85. Percentage of F1 *V. Viviparus* snails, from each treatment group, with encysted parasite infections. Snails sampled in September 2006, after developmental exposure to RW, RW+S, LM or HM.

	RW	RW+S	LM	HM
F1 males	0	0	100%	95.7%
F1 females	0	10%	94.7%	100%

The severity of parasitic infection (number of parasites per section) was measured using a parasite score. The severity of parasite infection increased with dose, in both male and female snails (Figure 55). Statistical analysis (Kruskal-Wallis test) found that there were significant differences, between treatments, in the mean parasite score for *V. viviparus* F1s (male P <0.001, female P <0.001) . Post hoc analysis found that snails exposed to RW and RW+S had significantly lower parasite scores than snails exposed to LM (male P <0.001, female P <0.001) or HM (male P <0.001, female P <0.001). There were no significant differences in parasite scores between *V. viviparus* snails exposed to HM and LM (male P = 0.398, female P = 0.061), or between snails exposed to RW and RW+S (male P = 1.000, female P = 0.683).

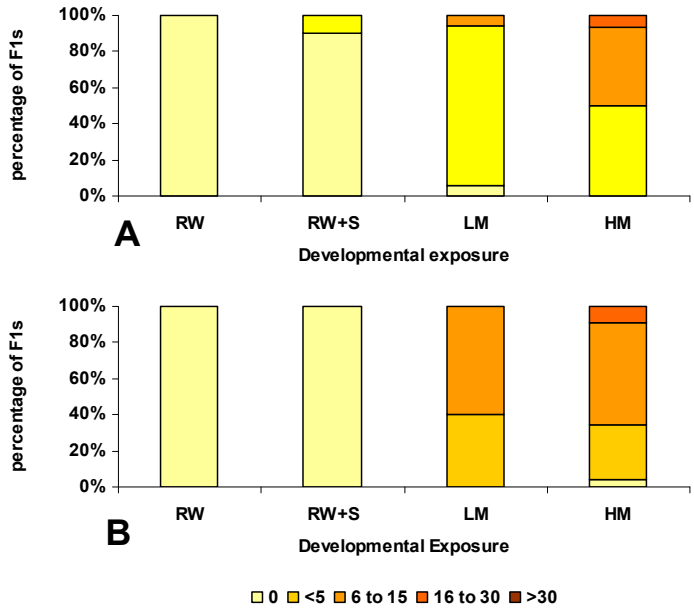


Figure 55. Histograms of encysted parasite infections in F1 *V. viviparus*. Snails were examined after exposure to RW, RW+S, LM or HM during their development. Histogram of the percentage of, (A) female and (B) male, V.viviparus with different intensities of parasite infection. 5 sections per snail were analysed. Pale yellow indicates no parasites found, yellow indicates less than 5 parasites per section, light orange indicates 5-15 parasites per section, dark orange indicates 15-30 parasites per section, brown indicates more than 30 parasites per section.

Effects of exposure to estrogenic mixtures on F1 *V. viviparus* survival, growth, reproduction and development following depuration

Survival

The survival of snails over winter was highest in the group that had been exposed to HM, compared to RW and LM treatments. Fifty-three percent of hatchlings survived in the RW treatment (273 individuals), 33% (66) survived from the LM treatment and 75% (63) from the HM treatment. Over the F1 breeding study, survival was highest in the group that had been exposed to HM and lowest in the group exposed to LM (RW, 84.8%; LM, 78.8%; HM, 96.7%).

Growth

At the beginning of the F1 breeding study, snails that had been exposed to LM or HM during their development were smaller than those from the RW treatment. Mean shell length ($P < 0.001$, ANOVA), shell aperture ($P < 0.001$, ANOVA) and total weight ($P < 0.001$, ANOVA) were significantly different between the three treatments. Post hoc analysis (LSD) found that snails exposed to RW were significantly larger than snails from the LM (shell length $P < 0.001$, shell aperture $P < 0.001$ and total weight $P < 0.001$) or HM (shell length $P < 0.001$, shell aperture $P < 0.001$ and total weight $P < 0.001$) treatments. The snails exposed to LM were also significantly larger than those exposed to HM (shell length $P = 0.026$, shell aperture $P = 0.022$ and total weight $P = 0.020$).

At the end of the F1 breeding study, females that had been exposed to LM during development had, on average, longer shells, larger shell apertures and greater total weight and soft body weight than the RW and HM treatment groups (Table 88). Statistical analysis (ANOVA) found there were no significant differences between the exposure groups.

Table 88. Mean size and weight (± SD) of F1 *V. viviparus* female snails at the end of an 18 week un-dosed breeding study. Prior to the measurements the snails had been exposed to RW, LM or HM during their development then depurated in river water. RW, n = 24; LM, n = 18; HM, n = 17.

	RW	LM	HM
Shell length mm	15.5 ± 1.3	16.0 ± 1.1	15.7 ± 1.1
Shell aperture mm	10.5 ± 0.7	10.6 ± 0.6	10.5 ± 0.7
Total weight g	1.0 ± 0.2	1.1 ± 0.3	1.0 ± 0.2
Soft body weight g	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1

The males that had been exposed to RW during their development were, on average, larger than those exposed to LM or HM (Table 89). However, statistical analysis (ANOVA) found there were no significant differences between the treatment groups.

Table 89. Mean size and weight of male *V. viviparus* F1 snails at the end of an 18 week un-dosed breeding study. Prior to the measurements the snails had been exposed to RW, LM or HM during their development then depurated in river water. RW, n = 4; LM, n = 7; HM, n = 15.

	RW	LM	HM
Shell length mm	15.5 ± 1.4	14.3 ± 1.5	15.1 ± 1.3
Shell aperture mm	10.1 ± 0.8	9.8 ± 0.9	10.1 ± 0.7
Total weight g	1.0 ± 0.3	1.0 ± 0.4	1.0 ± 0.2
Soft body weight g	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1

Reproduction

None of the F1 females from the three treatments released any neonates during the 18 week breeding study (Table 87). At the end of the study the brood pouches of female snails were dissected to count the number of embryos they were carrying. None of the females exposed to LM or HM were carrying any embryos (shelled or unshelled). In the RW treatment 12.5% of the F1 females were carrying embryos.

Table 87. The mean number of embryos (shelled or unshelled) in the brood pouches of F1 female *V. viviparus*, at the end of an 18 week F1 breeding study. V*.viviparus* had been exposed to RW, LM or HM during their development and then depurated in river water for eight months before being allocated into breeding groups.

	Shelled embryos	Un-Shelled embryos
River Water developmental exposed	0.04	0.33
Low Mix developmental exposed	0	0
High Mix developmental exposed	0	0

Gonadal histopathology of F1 *V. viviparus* following depuration

All surviving F1 snails were collected at the end of the F1 breeding study (after approximately one year of depuration) and analysed histologically. In the RW treatment group, 24 females and 4 males were collected, in the LM treatment, 18 females and 7 males, and in the HM treatment 17 females and 15 males. Five gonad sections were analysed per snail.

Effects on oogenesis

The mean oocyte number was highest in snails exposed to RW during their development. Oocyte numbers were reduced, in a dose-dependent manner, in snails exposed to LM and HM (Table 90). Statistical analysis (Kruskal-Wallis test) found there was a significant difference between the three treatment groups ($P = 0.001$). Post hoc analysis (Mann-Whitney test) found snails that had been exposed to RW had significantly more oocytes than snails that had been exposed to LM ($P = 0.009$) and HM ($P < 0.001$).

Table 90. Mean oocyte number (± SD) per section of gonad in *V. viviparus* F1 female snails. The snails had been exposed to RW, LM or HM during their development then depurated in river water for approximately one year. Five gonad sections were analysed per snail. RW, n = 24; LM, n = 18; HM, n = 17. Shared letters (a, b) indicate there was no statistically significant difference between these treatments.

	RW	LM	HM
Mean oocyte number	4.0 ± 4.06	1.4 ± 2.4	0.8 ± 1.5
	a	b	b

Effects on spermatogenesis

The mean spermatogenesis score was lowest in snails that had been exposed to LM during development and was highest in snails exposed to RW (Table 91). Statistical analysis (Kruskal-Wallis test) found there was no significant difference between the three treatment groups (P = 0.572).

Table 91. Mean (± SD) spermatogenesis score per section of gonad in *V. viviparus* F1 male snails. Snails had been exposed to RW, LM or HM during their development then depurated in river water for approximately one year. Score of 1= no germ cells, 10 = full spermatogenesis in >70% of tubules. Five gonad sections were analysed per snail. RW, n = 4; LM, n = 7; HM, n = 15.

	RW	LM	HM
Mean spermatogenesis score	9.95 ± 0.1	8.94 ± 1.83	9.61 ± 1.01

Parasitism

The majority of F1 snails that had been exposed to LM or HM during development had encysted parasite infections. None of the F1s from the RW exposure had parasites. The severity of parasite infection increased with dose (Figure 56). A Kruskal-Wallis test on the mean parasite scores found significant differences between treatments in both male and female snails; males (P = 0.001) and females (P <0.001). Post hoc analysis (Mann-Whitney test) found that snails exposed to RW had significantly lower parasite scores than snails that had been exposed to LM (male P = 0.039, female P <0.001) and HM (male P = 0.002, female P <0.001). The snails exposed to LM had statistically significant lower parasite scores than snails exposed to HM (male P = 0.014, female P = 0.041).

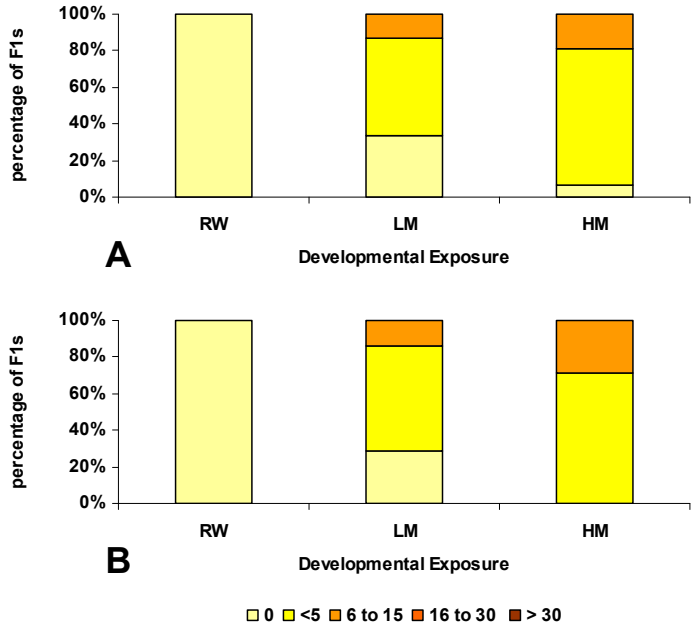


Figure 56. Percentage of F1 *V. viviparus* with parasitic infections. The snails were examined after exposure to RW, LM or HM during their development and depuration in river water for a year. (A) F1 females, (B) F1 males. The intensity of infection was scored from 0 encysted parasites per section (pale yellow) to a maximum of >30 encysted parasites per section (brown). Five sections of tissue were analysed per F1 snail.

2.6.3 Discussion

Survival of F1 snails, of both species, was very low in the RW+S tank during the exposure. This was also observed in the adults. However, the exposure had different effects in the two species. In *V. viviparus*, 49% of F1 snails exposed to RW+S had shells over 10mm in diameter, compared to 80% of the snails exposed to RW, 64% of the snails exposed to HM and 60% of the snails exposed to LM. In contrast, the F1 *P. corneus* in the LM and HM tanks were smaller than those from the RW or RW+S tank (44% of F1s from the RW tank had shells over 10mm in diameter, compared to 39% from the RW+S tank, 6% from the LM tank and 5% from the HM tank). The reasons for these differences in growth rate are unknown. It is interesting to note that exposure to alkylphenols or BPA during development has been reported to reduce the growth of juveniles in a number of species. Exposing tadpoles of two frog species (*Rana sylvatica* and *Rana pipiens*) to steroid oestrogens (E2 and EE2) or OP (0.25-10μM) reduced tadpole body size (Hogan et al. 2006). In the Japanese Medaka (*Oryzias latipes*) post–fertilisation exposure to OP (50μg/l) or BPA (200μg/l) significantly reduced growth (Knoor and Brunbeck, 2002; Ramakrishnan and Wayne, 2008). Both BPA (0.2-20ppb) and NP (0.2-2.0ppb) have been reported to significantly reduce growth in young viviparous swordtail fish (*Xiphophorus helleri*) and mixtures of the two chemicals were reported to inhibit growth more strongly than exposure to a single chemical (Kwak et al. 2001).

The survival of F1 *P. corneus* over winter was reduced in a dose-dependent manner. Among the F1s that had been exposed to HM during their development, only 2% survived the winter, 6% survived from the LM treatment and 19% survived from the RW treatment. The high mortality in the LM tank was probably due to the very small size/early stage of development of the snails, which was itself probably due to the high population density in this tank. However, during the F1 breeding study, the snails that

had been exposed to LM during development had twice the survival rate of snails exposed to RW. The high mortality over winter meant that only four F1 snails from the HM treatment were available for the F1 breeding study.

There was no dose-dependent trend in the survival of F1 *V. viviparus* over winter. In the RW tank 53% (273 snails) survived until the spring, compared to 33% (66 snails) from the LM tank and 75% (63 snails) from the HM tank. The high survival rate of *V.viviparus* that had been exposed to HM was in sharp contrast to the *P. corneus* survival rate. The overall density of snails in the HM tank was quite low (1.3l/snail) at the beginning of winter, compared to the RW (0.8l/snail) and LM (0.3l/snail) tanks. Competition for resources (e.g. dissolved oxygen and food) may have been less in the tanks where survival was the lowest.

During the breeding study none of the F1 female *V. viviparus* released any offspring. This is, perhaps, not surprising as the F1 females were roughly one third of the size of the adult females that were collected for the first breeding study, at the start of the experiment. However, at the end of the study, when the snails were large enough to be mature (15-16mm in shell length), none of the females exposed to LM or HM were carrying any embryos. In comparison, 12.5% of the females that had been exposed to RW were carrying embryos. *V. viviparus* has a long gestation period and animals need to mate in the previous autumn for females to harbour embryos the following spring. This may be a key factor in explaining the results. In contrast, the depurated *P. corneus* all reproduced during the study. The snails that had been exposed to LM laid more eggs per snail, over the entire breeding study, than snails exposed to the RW. In the HM treatment, although the snails began laying eggs at week 8 along with the RW and LM snails, they laid very few (4.3) eggs per snail. It appears that the long day length stimulated all the groups of snails to lay eggs during this time, even those negatively affected by chemical exposure. Joosse (1984) reported similar findings in *L. stagnalis* that were subjected to reduced temperatures and starvation. This highlights that, in these snails, the drive to reproduce overrides all others.

Histological analysis of the gonads of male and female *V. viviparus*, in the autumn after exposure, found reduced oocyte numbers and lower spermatogenic scores in snails that had been exposed to LM and HM, compared with snails from the control tanks. This may have affected their ability to mate. In the *V.viviparus* males spermatogenesis recovered after a year of depuration, but female oocyte number were still significantly reduced in snails that had been exposed to LM and HM, compared to the RW treatment. This demonstrates that oogenesis is negatively affected by developmental exposure to the chemicals and this effect persists even after a long period of depuration.

In addition to reduced reproduction and reproductive potential, the *V. viviparus* exposed to the oestrogenic mixtures had higher levels of encysted (metacerial digenea) parasite infection than the control snails. This was observed in both the adults and F1s. The F1s exposed to LM and HM had significantly higher infection intensities (parasite score) than snails exposed to RW or RW+S. The depurated F1s showed a similar trend. In addition, the snails exposed to HM had significantly higher parasite scores than the snails exposed to LM, indicating a dose-dependent increase in infection rate. Among the snails exposed to oestrogenic mixtures there was a change in the sex-related infection rate between the F0 and F1 generations of snails. Jezewski (2004) reported that female *V. viviparus* had higher parasite infection rates than males. A similar trend was observed in the adult *V. viviparus*. However, in the F1s exposed to oestrogenic mixtures this trend was reversed and the F1 males had a higher intensity of infection than the F1 females.

There is evidence that oestrogen and oestrogenic chemicals have an impact on mammalian immune function. Rodent studies have demonstrated that environmental oestrogens, such as OP, can promote splenic lymphocyte cell death (Nair-Menon et al. 1996) and BPA can modulate lymphocyte proliferation, lower concentrations stimulate and higher concentrations inhibit proliferation (Jontell et al. 1995). BPA has also been reported to alter macrophage function in rodents (Segura et al. 1999). Field studies of the freshwater bream (*Abramis brama*) found parasite infections (*L. intestinalis*) were elevated at a heavily polluted site (Hecker and Karbe, 2005). Caged studies of roach (*Rutilus rutilus*) reported that there were increased digenean infections downstream of Kraft mill effluent (Jeney et al. 2002). Roach exposed to treated sewage effluent displayed immunotoxic and genotoxic effects at low effluent concentrations that did not have classic ED effects (male vitellogenin stimulation, intersex; Liney et al. 2006). Recently, Filby et al (2007) exposed fathead minnows to treated sewage effluent and reported genotoxic effects and modulated immune function, including reduced lymphocytes indicative of immnosuppression. Exposure of fathead minnows to EE2 (in the same test system) had no effect on the immune system. In the European eel (*Anguilla anguilla*), experimentally exposed to PCBs, normal antibodies to the nematode *Anguillicola crassus* were not produced and infection rates were higher (Sures and Knopf, 2004). Several studies have reported lowered immune function and increased parasite infection in amphibians exposed to mixtures of pesticides (Christin et al. 2003; Hayes et al. 2006) and agricultural runoff (Kiesecker, 2002). Kiesecker (2002) reported increased digenea metacercariae (*Ribeiroia sp.*) and reduced numbers of eosinophils circulating in the blood of wood frogs (*Rana sylvatica*) with increasing exposure to agricultural runoff. The parasitised frogs were smaller than similarly exposed, but uninfected, frogs. Leopard frogs (*Rana pipiens*) exposed to a mixture of pesticides were found to be immunosuppressed (Hayes et al. 2006). Both control and exposed frogs tested positive for flavobacteria, but only frogs exposed to the pesticide mixture showed signs of pathology (Hayes et al. 2006). These results demonstrate that certain chemicals can modulate the immune system of vertebrates. This effect may also be relevant to *V.viviparus,* a long-lived freshwater invertebrate.

3. Objective 2a: Laboratory cultures

3.1 Objective

The objective of these experiments was to establish the conditions required for survival and reproduction of the species used in the mesocosm experiments, with the intention of eventually conducting laboratory-based exposures. Understanding how snails behave under pristine conditions in the laboratory is an essential first step that will allow any deviations from normal behaviour to be recognised when exposing snails to endocrine active substances. Once snails are successfully breeding in the laboratory, long-term cultures must be held to determine whether there is any seasonality in their behaviour (despite laboratory conditions remaining constant). Certain behaviours can cycle independently of external stimuli and these background changes must be understood. The aim of Objective 2 was to assess the suitability of native molluscs for culture in the laboratory.

3.2 Prosobranchs

V. viviparus and *B. tentaculata* were brought back to the Environment Agency Laboratory in Waterlooville and established in tanks of artificial pond water (see below). Attempts to establish prosobranch cultures were generally unsuccessful. A range of foods were offered (fish flake, fish pellets, lettuce, dried milk) that were not taken by the snails. *V.viviparus* and *B.tentaculata* have a sedentary mode of life. They prefer to filter feed over any form of searching and grazing, so the alga *C.vulgaris* was readily taken. However, this may not have been sufficient for the survival of the larger *V.viviparus*, as the adults reproduced for a short period, but died within three months. The juveniles grew well on the algal diet until they reached 6-8mm in diameter, but then died. This suggests that as the animals grew larger and reached sexual maturity they needed a more concentrated food source. In their natural riparian environment, *V. viviparus* spread a mucoid 'net' to catch particulate matter. This food collection procedure could not be reproduced in the laboratory.

There was more success with the alternative prosobranch, *B. tentaculata*, whose smaller size seemed to allow a better rate of survival and reproduction on the algal food supplied. Adults lived for up to six months in the laboratory and laid egg masses consistently. However, the tiny juveniles (approximately 1mm in diameter) seemed unable to access the algal cells and, as with the adults, did not take any form of highly ground particulate or liquid fish food. Therefore, it was not possible to establish an F1 generation. It is probable that both the juveniles and the adults graze, to some extent, on bacterial films, which it may be possible to establish in a laboratory in appropriate conditions. It was noted that individuals left in static, uncleaned tanks for long periods of time, laid more eggs than individuals maintained in an alga-fed culture. This is not a viable option for feeding snails during chemical testing, as the bio films effectively degrade organic compounds and animals cultured for use in tests should be fed using the same regime as used during the test.

3.3 Pulmonates

The pulmonate *P. corneus* was the only species where F1 adults could be raised to sexual maturity in culture. Consequently, the culture work described here will focus on this species. In terms of suitability for laboratory cultures, pulmonates have the advantage of being active-feeding opportunistic omnivores and of respiring at the water surface, which makes them better able to withstand semi-static laboratory conditions. The objective was to establish the parameters necessary for long-term survival of adults in good condition and for consistent reproductive activity. This includes establishing; the type and quantity of food required; any density dependent characteristics; water quality, flow rate or frequency of change requirements; and environmental requirements such as light and temperature. Finally, the intention was to achieve an acceptable rate of juvenile growth and development to sexual maturity, at least to F1, but preferably to F2, to ensure completion of the entire life cycle in the laboratory.

3.4 Methods

The animals were obtained from a biological supply house (Blades, Kent). On arrival in the laboratory they were introduced into 40l glass tank containing dechlorinated tap water and a selection of pondweed (from the same supplier). Snails were held at 20^{o}C under constant light to acclimate for at least 2 weeks.

Snails were sorted into size groups (<15mm, 15–18mm and >18mm along the longest axis of the shell) with no more than 50 animals in each group. Groups of 4 snails were then transferred into 10 x 10l tanks; the tanks were 15 cm wide, 30 cm long and 30 cm high. The tanks contained 8l of artificial pond water (APW), made to the following recipe:

Stock solution of 294g/l $CaCl_2.2H_20$, add 2ml/l to make 588mg/l media.
Stock solution of 123.25g/l $MgSO_4.7H_2O$, add 1ml/l to make 123.25mg/l media.
Stock solution of 64.75g/l $NaHCO_3$, add 1ml/l to make 64.75mg/l media.
Stock solution of 5.75g/l KCl, add 1ml/l to make 5.75mg/l media.

The stocking density was initially 1 snail/2l water, in a semi-static system with weekly water changes. The tanks were continuously aerated (200ml/min) using filtered air. Animals were fed weekly, after each water change, with 0.25g/snail of fish flake (King British, UK) and 3-4 pellets of calcium supplement (Crab Cuisine, Hikari, Kyorin, Japan). The APW recipe (above) contained double the usual amount of calcium chloride to reduce the likelihood of shell-thinning in the snails.

3.4.1 Culture experiment 1. Establishment of optimum stocking density

The stocking density was first maintained at 1 snail/2l water. It was later increased to 1 snail/1.5l (6 animals in 9 litres of APW) and finally to 1 snail/l (9 animals in 9 litres APW). In all stocking density experiments, animals >18mm in the longest axis of the shell were used because animals in this size class consistently laid egg masses. The egg masses produced were counted and recorded weekly, prior to each water change. Any mortality of adults within the groups was recorded and dead individuals replaced.

3.4.2 Culture experiment 2. Establishment of optimum water renewal rate at different temperatures

In this experiment, the frequency of water change was increased to every 48 hours. The stocking density was 1 snail/l and the egg masses were collected either weekly or every two days. To examine the effect of temperature, this experiment was replicated at 15ºC and 20ºC to broadly represent spring/autumn and summertime seasonal temperatures, respectively.

3.4.3 Culture experiment 3. Establishment of optimum feeding regime

To determine the optimum amount of food required (i.e. snails fed to excess while maintaining water quality), pairs of snails in 8l of APW were fed (only) on fish flake at 4 different rates on a logarithmic scale between 0.01 and 10g/snail. The pH, dissolved oxygen and conductivity of the water were measured daily to determine the effect of any excess food on water quality.

3.4.4 Culture experiment 4. Hatching studies

During these trials, all *P. corneus* eggs were hatched in semi-static conditions (half the water was changed three times a week) in 500ml glass jars at room temperature. Dechlorinated tap water was used in the hatching studies. Hatchlings were initially fed three times a week (after each water change) on 'Liquifry' one and two (Interpet commercial food for fish hatchlings), and when larger, on crushed fish flakes, as detailed in the preceding section on culture conditions. Two trials were conducted to measure hatching and survival at both low and high population densities.

For the high density trial, egg masses were placed in ten 500ml jars to produce a range of egg numbers (between ~300 to 1000 eggs). The masses were left to hatch for two weeks, after which hatching success was assessed. At two months old, snails from were removed from their jars, counted and then all were moved to an 80l flow-through tank and fed ad libitum on fish flakes and organic lettuces. The time at which the first egg masses were produced by the young snails was recorded. For the low density trial, fewer eggs per 500ml jar were used (between ~80 and 100 per jar).

3.5 Culture Experiment 1: results

3.5.1 Culture experiment 1. Establishment of optimum stocking density

The mean number of egg masses produced/snail/week ranged between 3.3 and 4.5, at a stocking density of 1 snail/1.5l at 20^{o}C in semi-static conditions with weekly water changes. This was the approximate production rate of the snails observed throughout the year. Similar reproductive output was seen at the lower stocking density of 1 snail/2l. At even lower stocking densities, for instance 2 snails in 8-10l, the snails barely laid egg masses at all. Conversely, at the highest stocking density (1 snail/l)

there was a higher mortality rate, although the snails continued to lay egg masses. Therefore, the optimal stocking density was 1 snail/1.5l at 20°C.

3.5.2 Culture experiment 2. Establishment of optimum water renewal rate at different temperatures

The rate of egg mass production was unchanged when the frequency of water changes was increased from once a week to once every 48 hours, for snails stocked at the optimum density of 1 snail/1.5l. (Egg masses were still collected weekly). When this was repeated at 15°C, at the same stocking density, egg mass production was reduced to a mean value of 2.1 – 2.8 egg masses per snail per week.

The possibility that survival might be improved at the higher stocking density of 1 snail/l, with increased frequency of water changes and cooler temperatures was explored. Under these conditions, the high mortality rate that was observed previously did not occur. Most snails survived and were of healthy appearance and egg mass production remained at approximately 2 masses per snail per week. When the frequency of water changes was reduced to one per week, at this temperature, egg mass production was considerably reduced (0.77 – 0.88 egg masses per snail per week).

Summary data for culture experiments 1 and 2 are presented in Table 95. Egg mass production was affected by temperature; snails held at 15ºC typically producing less egg masses per week than those held at 20ºC. The optimum stocking density is 1 snail/1.5l in a semi-static system at 20ºC with weekly water changes, or 1 snail/l at 15ºC, if 48 hour water changes or flow-through water replacement are used.

Table 95. Summary of egg mass production at different temperatures, water change regimes and stocking densities.

Density	Temp	Water Change	Tanks	Monitoring period	Masses/snail/week +/- SEM
1 snail/1.5l	20	Weekly	10	4 weeks	3.96 ± 0.50
1 snail/2.0l	20	Weekly	10	2 weeks	4.04 ± 0.09
1 snail/1.5l	20	48 h	20	2 weeks	3.78 ± 0.34
1 snail/1.5l	15	48 h	20	2 weeks	2.46 ± 0.46
1 snail/1.0l	15	Weekly	6	3 weeks	0.84 ± 0.06
1 snail/1.0l	15	48 h	14	2 weeks	1.99 ± 0.07

3.5.3 Culture experiment 3. Establishment of optimum feeding regime

Production of egg masses was unaffected between feed rates of 0.1 and 1g/snail, every 2-3 days, as shown in Figure 58. Productivity was only affected at the 0.032g/snail rate, this rate does not appear to be sufficient to support egg-laying as no egg masses were laid.

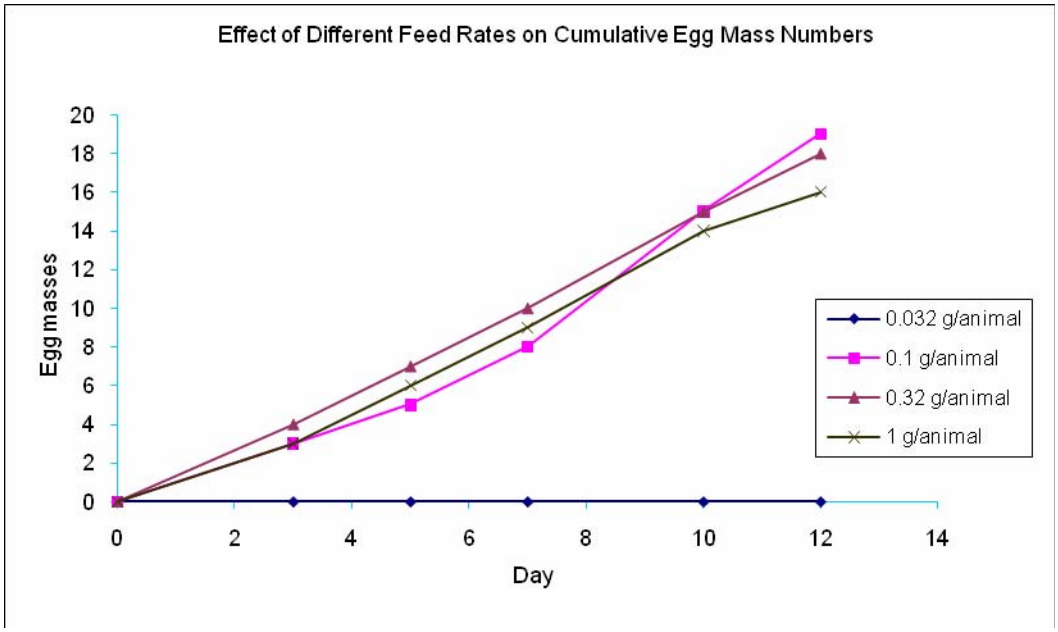


Figure 58. Cumulative egg mass production of *P. corneus* given different rates of feed per snail.

3.5.4 Culture Experiment 4. Hatching studies

Figure 64 shows there is a trend with decreasing stocking density for increased hatching success (from approximately 40% to generally >80%) and juvenile survival to two months old (although juvenile survival was never greater than 40%). The optimal time to maturity was 12 weeks (at low density with water temperatures between 19 and 23^{o}C).

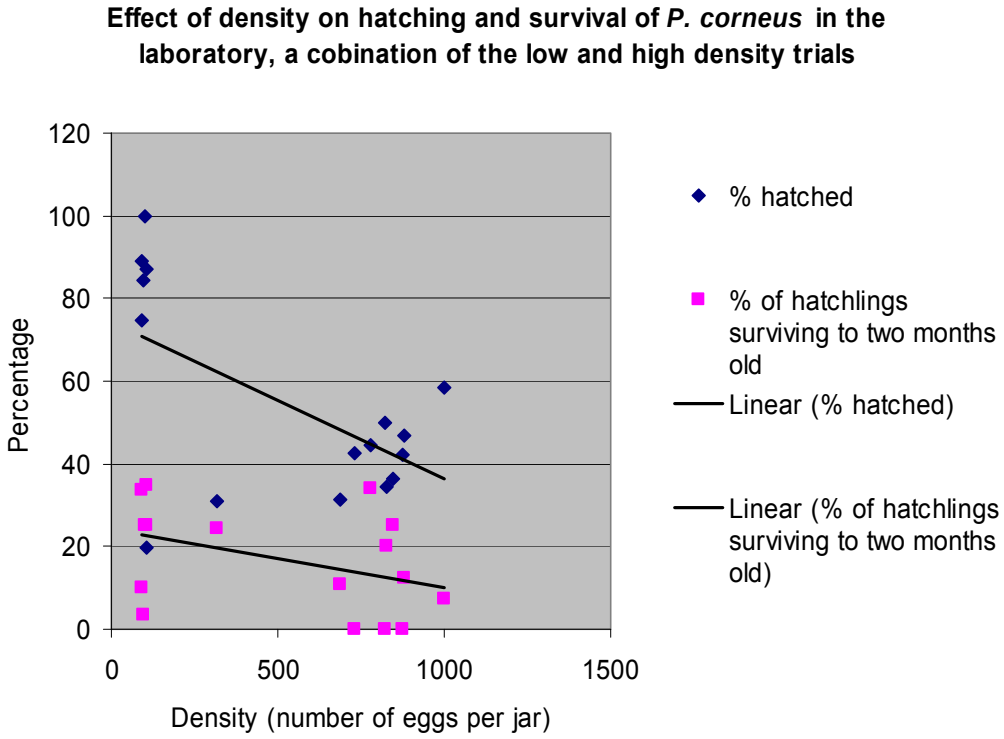


Figure 64. Combination of results from trials 1 and 2, Overall effect of density on hatching success and hatchling survival of *P. corneus* in the laboratory.

3.6 Discussion

The results of experiments with *P. corneus* demonstrate that this species can easily be maintained in the laboratory. This is in contrast to the two prosobranch species (*V. viviparus* and *B. tentaculata*) which were unsuccessful in establishing long term cultures. Stocking density, feed rate, water temperature and frequency of water changes were important factors that affected the snails' ability to lay eggs. Optimum feed rates for adult snails were between 0.1 and 0.32g/snail of fish flake, dependent on the frequency of water changes. For snails in culture, it is considered advantageous to supply calcium-enriched pellets, to reduce the likelihood of shell-thinning over the long-term, but this would not be required during chemical exposures. When feeding juveniles, a more accessible food supply than fish flake is necessary and both algal concentrate and Live Bearer Liquid Feed are acceptable. One snail per 1.5 litres of artificial pond water, with weekly water changes, was optimal. A higher density (1 snail/l) required cooler temperatures and more frequent water changes to avoid high mortality rates. It should be noted that it may be necessary to remove egg masses at intervals of less than a week, as egg mass production slowed when there were more than a dozen or so masses present on the surface area provided (approximately 0.25 m^3). Whether this is the result of a chemical cue released by the egg masses or due to the rate at which snails encounter egg masses as they move around the tank is not known.

In general, the number of eggs in each mass was much greater than has been reported in the literature. Costil and Daguzan (1995) reported that the mean number of *P. corneus* eggs per mass was 8 at 15°C, 14.8 at 20°C and 10.1 at 25°C. In these studies several times this amount was recorded. Another marked difference between these results and those of Costil and Daguzan is the total number of egg masses produced over a life span. They found between 17 (at 25°C) and 123 (at 20°C) egg masses were

laid per snail. These are very low numbers compared to this study where one group of six snails laid over 200 egg masses in one month. The marked differences seen between the productivity of the snails in this study and Costil and Daguzan's 1995 study may be due to regional differences (their *P. corneus* were from France and ours were sourced from the UK) or due to nutrition and/or density effects (Costil and Daguzan's snails were fed only on lettuce).

Population density significantly affects hatching success. A significant ($P < 0.001$) increase in hatching success was found in the lower egg density groups (~100 eggs/jar), compared to the high density groups (from 300-1000 eggs/jar). The mean hatching success rate, of the lower density replicates, was 80%, but the minimum hatching success (19.8%) was still far less than hatching success reported by Costil (1997). Costil reports that microorganisms may be advantageous in breaking down egg walls, thereby facilitating hatching. Thus, the low hatching success observed in the laboratory may be due to a lower bacterial load in dechlorinated tap water than in natural pond water.

It was not possible to rear the F1 snails at densities of 1 snail/l due to space constraints. As a consequence, large differences were observed between individuals in terms of their size and rate of maturity. It is likely that the culture was overstocked to some degree and that competition slowed the development of the weaker individuals. If this were allowed to continue in the long-term, it could affect the population of snails used to supply a test, perhaps making those selected for use more competitive and possibly less sensitive to challenges.

4. Objective 2b: laboratory exposures

4.1 Two concurrent exposures of *P. corneus* to E2 in simulated 'Summer' and 'Autumn' conditions Objective

Having established that *P.*corneus was sensitive to estrogen exposure and amenable to culture, the objective of this experiment was to make controlled exposures to E2. One exposure was made under simulated summer temperature and day-length conditions (20^{o}C and 16 hours light/8 hours dark) and the other in a simulated autumn (15^{o}C and 12 hours light/12 hours dark) .

4.1.1 Materials and Methods

Experimental set-up

Two windowless, air-conditioned rooms with insulated walls and doors were programmed to hold temperatures of 15°C and 20°C, respectively. The maximum and minimum temperatures in the rooms were recorded daily.

The shelves where the experiments were conducted were each lit by two Phillips 'Fluotone' strip bulbs. The emitted cool white light had a lux value of 4000, measured at the height of the water surface in the test tanks. The photoperiod was automatically controlled and set at 12 hours light and 12 hours dark in the room at 15 °C, and 16 hours light and 8 hours dark in the room at 20 °C. The functioning of the photoperiod controls was checked monthly by manual forwarding of the control clock to ensure switching.

The snails were housed in groups of 6 in covered plate glass tanks with a working volume of 9l. Each tank was allocated a consecutive number. The tanks were aerated at a rate of 200ml/min (see Figure 69). The tanks were filled with Artificial Pond Water (APW) made with carbon filtered, reverse osmosis water that had been allowed to equilibrate to the appropriate temperature before adding salts according to the recipe in objective 2a. At 48-hour intervals, the media in the tanks was drained down to 3l, faeces and excess food were removed by vacuum siphon and the tanks were replenished with 6 litres of fresh medium. The water change process was divided into fortnightly 'cycles'. Every fortnight the snails were removed into a correspondingly numbered vessel containing approximately 1l of their tank water. Their tanks were then completely drained, washed using chlorinated tap water and a clean cloth and filled with fresh medium. The shells of the snails were gently cleaned (with fingertips) if necessary prior to their return to their clean tanks. This was to avoid seeding the fresh medium with appreciable amounts of algae and bacteria.

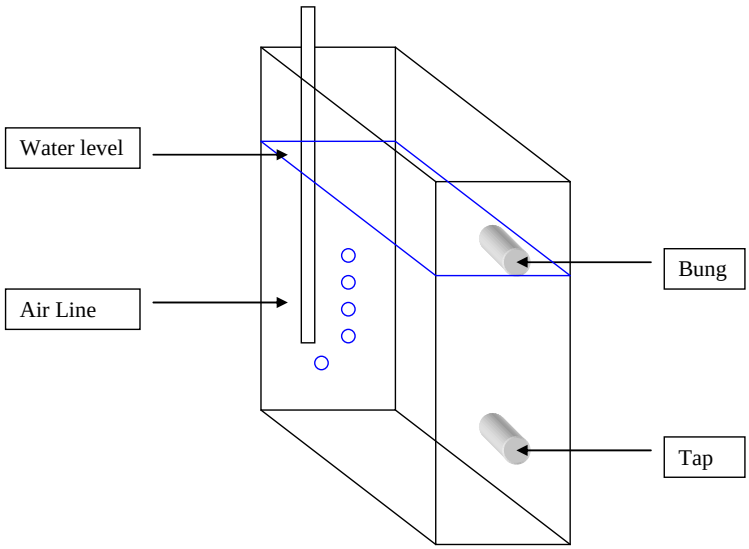


Figure 69. Diagram of an exposure tank (15cm width x 30cm height x 30cm depth; water depth 24cm). The tanks were constructed using silicon sealant and had an outlet tap approximately 8cm from the bottom, which was made from ABS and polythene plastics.

Prior to, and after, each complete or partial water change, a 100-200ml water sample was taken from each control tank and the pH, dissolved oxygen and conductivity measured. The hardness of each new batch of medium was measured by titrating against EDTA in the presence of excess ammonia. At the end of each cycle, the hardness of all control tanks was measured.

After a 4 week baseline period, the 8 week exposure to E2 began. A 1 ml aliquot, of a 10mg/l solution of E2 in methanol, was added to a 1l glass conical flask containing approximately 250ml of 5mm diameter glass beads. The beads and liquid were mixed and the methanol allowed to evaporate for 30 minutes under a flow of filtered air. One litre of reverse osmosis, filtered water was divided into 4 - 6 aliquots and each aliquot was added, in turn, to the dried beads, shaken vigorously and tipped into a collection vessel. This provided a working solution of 10ug/l E2. Volumes of 1, 10 or 100ml of the working solution were then made up to 10 litres with APW, to make the nominal test concentrations of 1, 10 or 100ng/l of E2 for use in the tanks. The numbered tanks were randomly allocated treatments (control, 1, 10 or 100ng/l nominal E2).

Test organisms

Because of the difficulty of finding a single supplier who could deliver the numbers of *P. corneus* snails required, approximately half of the snails were obtained from 'Sciento' Biological Supply House (Manchester) and the other half from Queensborough Fish Farms (Staines). Snails from both suppliers were received over a two-week period in late April/early May. They were divided into two equal groups on arrival and placed in 100l tanks containing APW, in the 15^{o}C or 20^{o}C constant temperature rooms. In this way, the snails from both suppliers were split equally across the rooms (~150 snails in each holding tank) and were allowed to freely intermix in their holding tanks. The mixed populations were allowed to acclimate for another week, while becoming accustomed to consuming fish flake food (King British, UK).

At the start of the experiment, snails were drawn from the holding tanks and individuals were weighed and measured along their longest axis. Snails that were ≥18mm long

(sexually mature) were then randomly allocated to their treatment tanks, using a random number table, until each tank contained 6 individuals. A record was kept of the details of each animal allocated to each treatment tank. The snails were fed on fish flake food after each partial media change at a rate of 0.25g/snail (the total weight of food added was adjusted for each snail death).

Reproduction rates were very variable between the individual groups of snails and, consequently, a baseline period was used to determine the relative productivity of each group. During the baseline period (the first four weeks of the experiment) a provisional minimum quality threshold was set for the number of masses laid/adult/48 hours (mean = 0.5) in order to establish that the snails were behaving as expected. At 20°C all the groups passed this threshold. However, at 15°C four groups were close to, or below, this minimum and so these four tanks were removed. At 20°C, all 20 tanks were allocated evenly between the control and three test concentrations, giving 5 replicates per treatment, but at 15°C only 16 tanks were available, giving 4 replicates per treatment.

E2 stability test

Before starting the exposure experiment an E2 stability study was performed. This used a single tank, containing 6 snails, for each E2 concentration - 1, 10, 100 and 1000ng/l. The snails were fed only on fish flake food at a rate of 0.25g/snail, as described for the exposure experiment. The stability test was conducted at 20°C with 16 hours light and 8 hours dark, as bacterial degradation of E2 was expected to occur more quickly under these environmental conditions (worst case scenario). Samples were taken from each tank at time zero, 3, 9, 21 and 45 hours, which provided degradation time intervals of 3, 6, 12 and 24 hours between samples.

Sampling

At fortnightly intervals, at the mid-point of each cycle, aliquots for chemical analysis were taken from each tank, before and after the partial water change. The samples for each treatment, at each temperature, were pooled.

At each 48-hour partial water change, all egg masses were removed and the eggs in each mass counted and any egg abnormalities noted. The egg masses from each tank were air dried for 2-5 days and then collectively weighed. On one occasion during each cycle, egg masses from each tank were counted and transferred to 100ml crystallising dishes that contained medium with the appropriate chemical dose. These were left at 20°C for 14 days to hatch out (the medium was changed every 48 hours). At the end of this period, the numbers of hatched and unhatched eggs were counted.

At the end of the treatment, all snails were placed in a 5% solution of magnesium chloride for approximately 2 hours to relax their tissues. All the snails were weighed and measured along their longest axis. Their shells were then removed and the soft body parts weighed. The reproductive tract (ovo-testis, the hermaphroditic ducts, the albumin gland, the prostate, sperm ducts and penile complex, the oothecal gland, oviducts and vagina) were dissected out and weighed.

4.1.2 Results

E2 stability test

Figure 71 shows that E2 degraded over time under the test conditions (r^2 was above 0.9 in all cases).

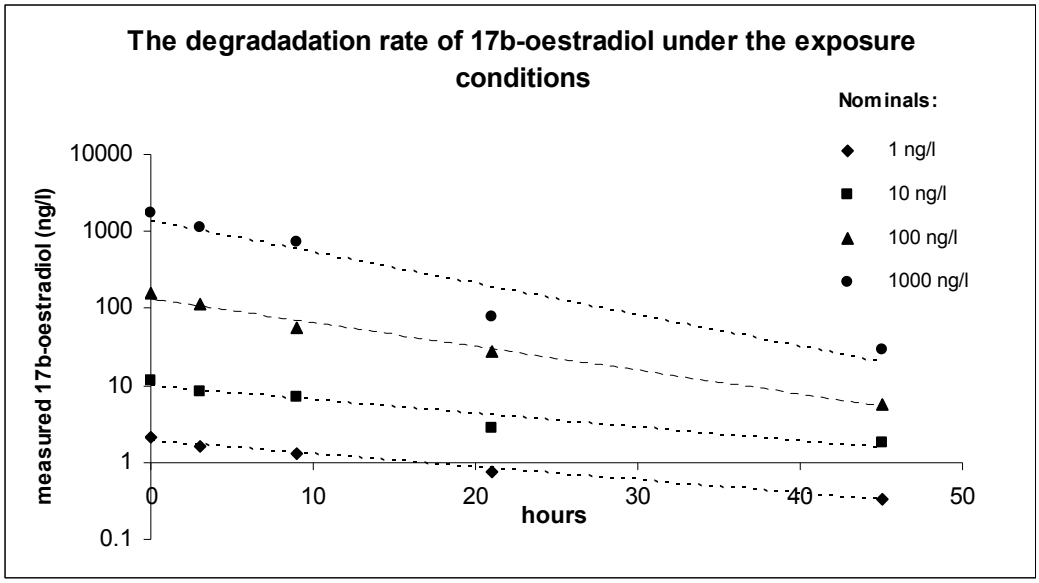


Figure 71. The measured degradation rate of E2 at 20°C under the conditions used in the exposure test.

Chemical analysis

The results of the fortnightly E2 analysis performed during the 8-week exposure are set out in Table 106. The mean concentrations are also given.

Table 106. The results of the E2 and E1 analysis performed prior to (PRE) and after (POST) each media change during the 8-week exposure of *P. corneus.* ND = No Data presented due to analytical error (cycle 2; reported E2 values 10-fold too high, but E1 normal, indicating a calculation or dilution error by analytical laboratory) or contaminated glassware used to collect the water samples (cycle 3) which resulted in control values in all cases of ~ 1ng/l. Therefore, the measured concentrations in the controls in cycle 3 were subtracted from the measured values in the exposure tanks to provide a corrected measure.

	E2 (ng/l)			E1 (ng/l)		
1st Exposure Cycle *(8th – 21st June)*	PRE	POST	Mean	PRE	POST	Mean
15°C, Control	<0.2	<0.2	<0.2	0.392	<0.2	0.196
15°C, 1ng/l nominal	0.390	0.896	0.643	1.55	0.442	0.996
15°C, 10ng/l nominal	ND	ND	-	ND	ND	-
15°C, 100ng/l nominal	24.0	122	73.0	151	4.8	97.4
20°C, Control	<0.2	<0.2	<0.2	0.598	<0.2	0.299
20°C, 1ng/l nominal	0.372	1.72	1.05	1.39	0.818	1.10
20°C, 10ng/l nominal	2.20	11.4	6.80	9.54	3.3	6.42
20°C, 100ng/l nominal	21.0	122	71.5	106	41.3	73.7
2nd Exposure Cycle *(22nd June – 5th July)*	PRE	POST	Mean	PRE	POST	Mean
15°C, Control	0.744	0.204	0.474	0.432	<0.2	0.216
15°C, 1ng/l nominal	0.826	0.952	0.889	0.908	0.356	0.785
15°C, 10ng/l nominal	12.8	14.7	13.8	6.32	2.58	4.45
15°C, 100ng/l nominal	150	160	155	77.6	27.2	52.4
20°C, Control	0.690	0.718	0.704	0.502	0.262	0.382
20°C, 1ng/l nominal	ND	ND	-	ND	ND	-
20°C, 10ng/l nominal	12.6	16.9	14.8	9.06	2.52	5.79
20°C, 100ng/l nominal	117	178	148	66.2	33.6	49.9
3rd Exposure Cycle *(6th – 19th July)*	PRE	POST	Mean	PRE	POST	Mean
15°C, Control	ND	ND	-	ND	ND	-
15°C, 1ng/l nominal	0.716	1.06	0.888	0.970	0.364	0.667
15°C, 10ng/l nominal	0.606	1.58	1.09	4.80	2.64	3.72
15°C, 100ng/l nominal	23.2	38.4	30.8	64.6	29.8	47.2
20°C, Control	ND	ND	-	ND	ND	-
20°C, 1ng/l nominal	2.38	1.26	1.82	1.07	0.414	0.742
20°C, 10ng/l nominal	3.38	3.86	3.62	5.52	2.94	4.23
20°C, 100ng/l nominal	21.2	43.2	32.2	44.8	20.8	32.8
4th Exposure Cycle *(20th July – 2nd August)*	PRE	POST	Mean	PRE	POST	Mean
15°C, Control	<0.2	<0.2	<0.2	<0.2	2.76	0.138
15°C, 1ng/l nominal	<0.2	0.514	0.257	1.14	0.454	0.797
15°C, 10ng/l nominal	0.816	5.14	2.98	5.62	2.48	4.05
15°C, 100ng/l nominal	24.8	100	62.4	58.2	23	40.6
20°C, Control	<0.2	0.288	0.114	<0.2	0.362	0.181
20°C, 1ng/l nominal	0.542	0.318	0.430	0.332	0.750	0.541
20°C, 10ng/l nominal	1.59	7.26	4.43	5.38	2.44	3.91
20°C, 100ng/l nominal	13.8	90.0	51.9	59.4	19.3	48.9

The standard deviations for these figures are high, but when the concentrations are compared, all are significantly different from each other:
Comparison of control and nominal 1ng/l; p = 0.021 (t-test)
Comparison of nominal 1ng/l and 10ng/l; p = 0.003 (Mann-Whitney Test)
Comparison of nominal 10ng/l and 100ng/l; p = 0.002 (Mann-Whitney Test)

For each cycle, the mean pH was between 7.0 and 8.0, the mean dissolved oxygen was >80% ASV, the mean conductivity was <1.1mS/cm and the mean hardness was

between 390 and 440 mg/l $CaCo_3$ equivalent. Table 107 shows the minimum and maximum temperatures recorded for each cycle of the baseline and exposure periods. The temperature was also measured manually, using a mercury thermometer with an error margin of 0.5°C, in samples taken from the test tanks to characterise water quality parameters. A mean temperature for each period, from these readings, is also given. Temperature did not deviate by more than 2°C from the nominal in either of the constant temperature rooms. However, on one occasion (3rd exposure cycle) the switching mechanism for the 15°C room failed and the room temperature rapidly increased to 24.5°C. The malfunction was rectified within two hours and the behaviour of the snails appeared unaffected. The automatically recorded temperature data from this event have been omitted. Apart from on this occasion, the temperatures of the constant temperature rooms did not come closer than 3.6°C of each other.

Table 107. The mean, minimum and maximum temperatures experienced by the exposure tanks in each constant temperature room, for each cycle of the experiment.

	Temperature (°C)		
Experimental Cycles at 20 °C	**Mean**	**Minimum**	**Maximum**
1st Baseline Cycle *(11th - 24th May)*	21.1	20.1	21
2nd Baseline Cycle *(25th May - 7th June)*	20	19.9	20.9
1st Exposure Cycle *(8th – 21st June)*	19.7	20.1	21
2nd Exposure Cycle *(22nd June – 5th July)*	20	20.3	21.1
3rd Exposure Cycle *(6th – 19th July)*	20.6	19.9	21.3
4th Exposure Cycle *(20th July – 2nd August)*	20.2	19.2	21

	Temperature (°C)		
Experimental Cycles at 15 °C	**Mean**	**Minimum**	**Maximum**
1st Baseline Cycle *(11th - 24th May)*	15.7	13.9	15
2nd Baseline Cycle *(25th May - 7th June)*	15.1	14.1	15
1st Exposure Cycle *(8th – 21st June)*	15.3	14.4	15.1
2nd Exposure Cycle *(22nd June – 5th July)*	15.4	14.4	15
3rd Exposure Cycle *(6th – 19th July)*	15.7	14.2	15.6
4th Exposure Cycle *(20th July – 2nd August)*	15.4	14.2	15.1

Effects of E2 exposure on *P.corneus*

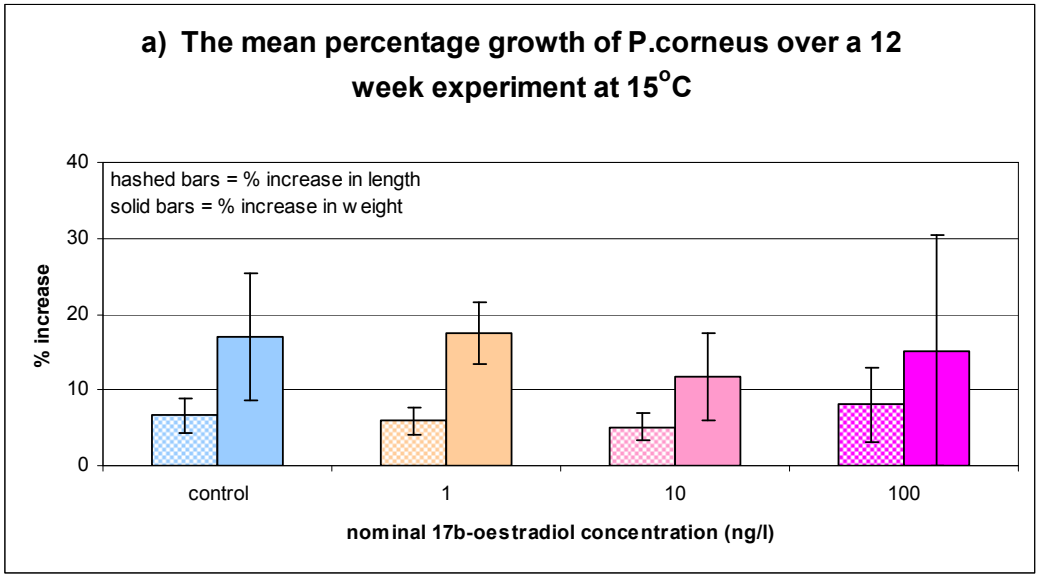
Mortality

In the experiment performed at 15°C, the mortality over the duration of the exposure was negligible. There was only one death across all of the control groups and likewise one death at each test concentration. In the experiment performed at 20°C the mortality rate was more significant. Three animals died across all of the control replicate groups and three at each test concentration (10% of the test organisms) up until the last week of the experiment, when a further three control animals died, taking the mortality rate of the control group to 20%.

Growth

At the start of the experiment there were no significant differences between the control groups and any of the treatment groups at either temperature (P >0.05, ANOVA). At

the end of the treatment growth was calculated in terms of percentage increase in length and weight (Figure 72). At 20°C, the smaller and lighter control group showed the smallest increases in length and weight, compared to the groups exposed to E2. At 15°C, the group exposed to the highest concentration of E2 (78ng/l) grew the most in terms of length, but did not show a corresponding increase in weight. None of these differences were statistically significant (P >0.05, ANOVA).



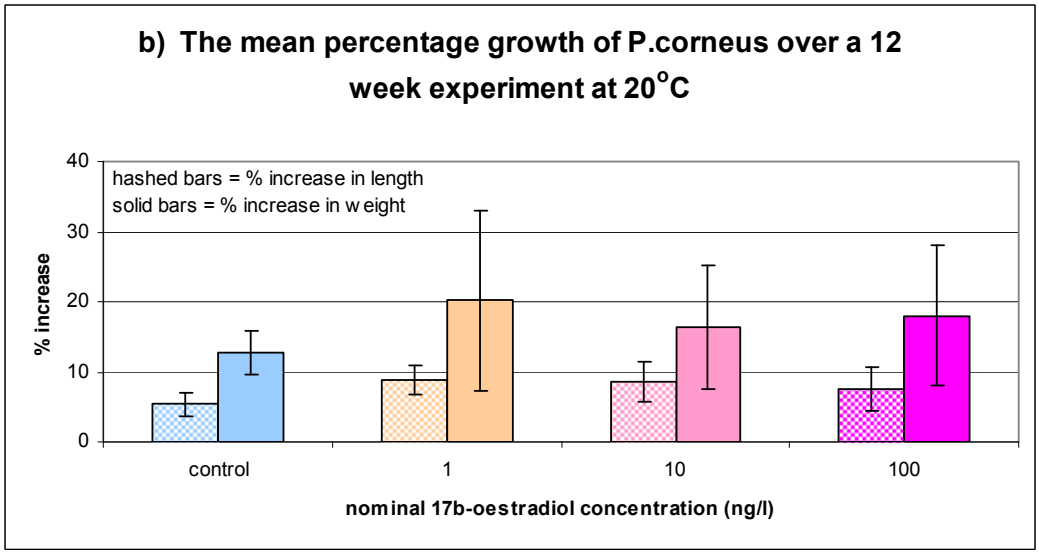


Figure 72. The mean percentage increase in length and weight of *P. corneus* at 15°C (a) and 20°C (b) over a 12 week long experiment.

At the end of the treatments the shells were removed from the animals and the soft tissues weighed. To give an indication of body condition factor (BCF) the ratio between the mean weight of each group with and without their shells was calculated. The reproductive organs were then dissected out and the ratio between the mean total body weight and mean reproductive organ weight was calculated for each group, to give a gonadosomal index (GSI). There were no significant differences between any control or exposure groups for either BCF or GSI, at either temperature (P >0.05, ANOVA).

Reproduction

The number of eggs produced by each group was adjusted for the number of surviving snails. Figures 73 and 74 show the mean number of eggs produced per surviving snail at 15°C and 20°C. At 20°C there was little evidence that egg production was affected by exposure to E2; egg production rates were similar in the baseline and in all treatments. In the pre-exposure period, egg production in snails at the higher temperature was nearly double (approximately 700 eggs per adult in 4-weeks) that from snails at the lower temperature (approximately 360 egg per adult in 4-weeks). Figure 74 shows that, as expected, the reproductive rate of control snails at the lower temperature (onset of 'autumn') was significantly reduced in comparison to the baseline period (paired t-test, $P = 0.031$). The same phenomenon occurred to a lesser extent in the 1ng/l and 10ng/l (nominal) treatment groups (the differences were significant at $P = 0.007$ and $P = 0.026$, respectively). There was little evidence of a decline in the reproductive rate of snails exposed to 100ng/l (nominal) E2. In these groups, there were no significant differences in the rate of oviposition between the baseline and exposure periods.

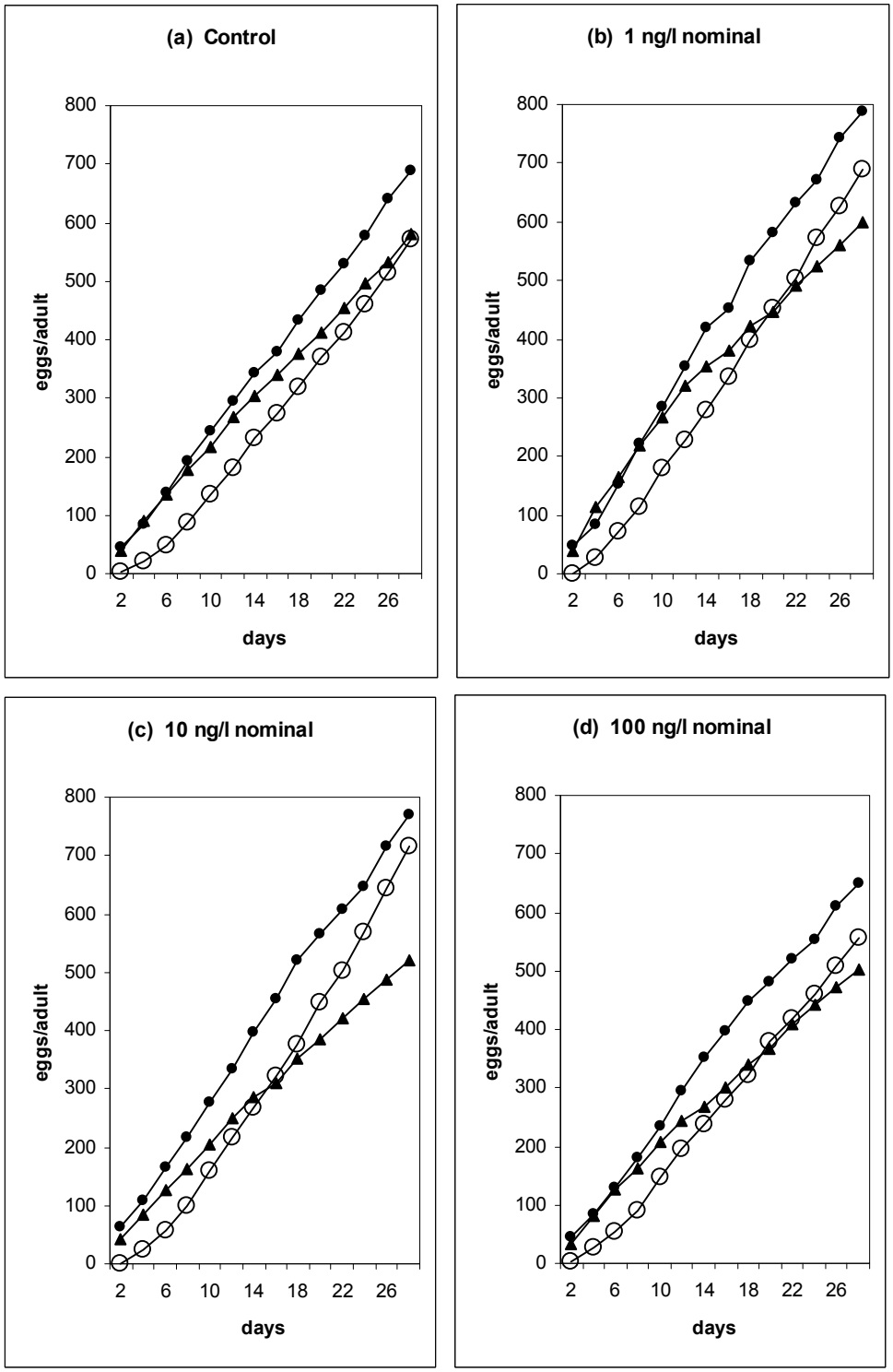


Figure 73 The mean number of eggs produced by surviving adult *P. corneus* over a 28 day exposure to two concentrations of E2 in the laboratory at 15°C with a 12 hour light, 12 hour dark photoperiod. Open circles represent the 4-week baseline period, black circles and black triangles represent the first and second 4 weeks of the exposure period respectively.

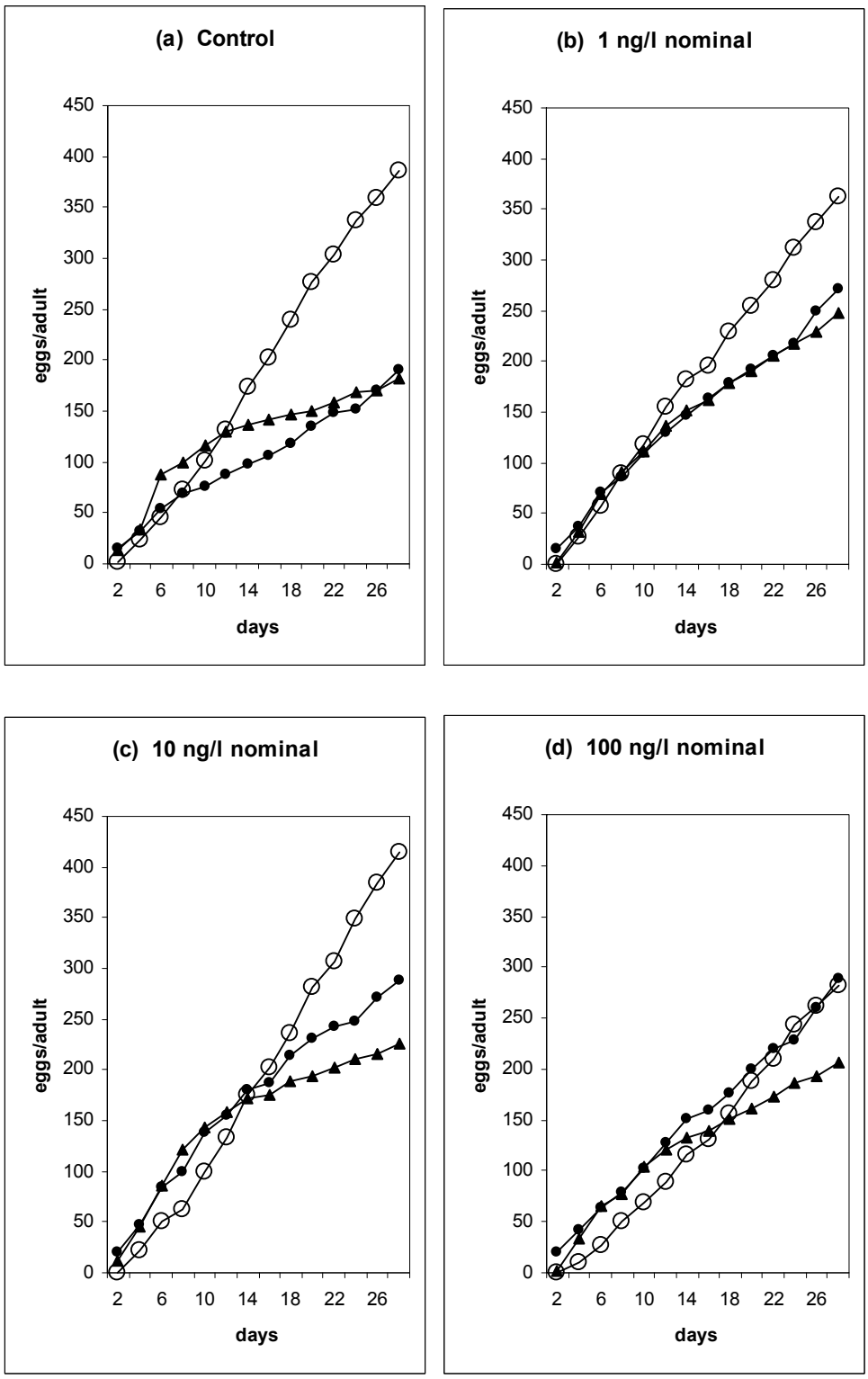


Figure 74 The mean number of eggs produced by surviving adult *P. corneus* over a 28 day exposure to two concentrations of E2 in the laboratory at 20°C with a 16 hour light, 8 hour dark photoperiod. Open circles represent the 4-week baseline period, black circles and black triangles represent the first and second 4 weeks of the exposure period, respectively.

Parasitism

At the end of the exposure, a few test organisms were found to have parasitic infections (6.5%). These animals were presumed not to have contributed to the reproductive population and the data were adjusted accordingly.

Egg mass characteristics

Dry Weight

The mean dry weights of the egg masses laid by the control and treatment groups were very similar (~ 6mg/mass). At 15^{o}C, the mean dry weight of the egg masses was more variable across the test groups, but generally heavier (up to 8mg/mass). There were no significant differences in the mean dry weights of the egg masses from the control and E2 exposed groups, at either temperature (P >0.05 in all cases, ANOVA).

Number of Eggs per Mass

At 20^{o}C, the mean number of eggs per mass was between 30 and 40 in the control and E2 groups. At 15^{o}C, the mean number of eggs per mass was similar. There were no significant differences in the mean number of eggs per mass between the control and E2 exposed groups, at either temperature (P >0.05 in all cases, ANOVA).

Egg abnormalities

Abnormalities that were likely to have affected the hatching success of the egg were recorded. These abnormalities included; eggs without an embryo, eggs with the embryo outside of the egg capsule and eggs with multiple embryos inside the egg. The percentage of abnormal eggs was calculated by dividing the number of abnormal eggs by the total number of unbroken eggs (broken eggs were excluded as it was not possible to tell whether these were normal or not) and multiplying by 100.

The mean abnormality rate in the control group was lower than in all the exposed groups at both temperatures. However, there were no significant differences from the control (P >0.05, Kruskal-Wallis test).

Egg hatching success

Once during each cycle of the experiment the number of eggs that successfully hatched after 14 days was counted. During the baseline period, at 20^{o}C, a mean hatching rate of 67.4% (SD = 25.1) was recorded at the first test, the rate dropped to only 15.4% (SD = 20.4) on the second occasion. At 15^{o}C, the hatching rate was 19.0% (SD = 22.8) on the first occasion and 6.27% (SD = 12.9) on the second. There was similar variation in hatching success from egg masses collected from all of the treatment groups during each cycle of the exposure. There were no significant differences, from the control, on any occasion (P >0.05, Kruskall-Wallis test).

4.1.3 Discussion

The stability study showed that the concentration of E2 declined in all treatments. The half life was notably shorter at higher concentrations, possibly due to more rapid

adaptation of bacteria where the energy source was more plentiful. The half-lives were too short to feasibly dictate the frequency of water changes and it was accepted that the snails would experience peaks and troughs in E2 concentrations over the, shortest practicable, water change cycle. Media was prepared and changed on alternate days, so as to allow the prepared media 24 hours to equilibrate. Although the degradation rate of E2 was high, the analytical results showed clear differences in the amount of E2 present in the treatment tanks.

Adult mortality rate was lower than in the mesocosm experiments. It is not clear why there was no increase in mortality in the highest E2 concentration, as was seen in the mesocosm, but this may be related to general stresses, which are less likely to occur in the 'optimal' conditions of a laboratory.

The oviposition rate in all groups was similar to the higher of the rates observed in the mesocosm experiment (up to 49.5 eggs per individual per 48 hours). In the simulated summer conditions, the rate was close to constant during the baseline and exposure periods in the control and treatment groups. There was a slightly slower start (possibly due to adaptation to the exposure system) and end (possibly due to a degree of senescence following a long period of intense reproduction). In the simulated autumn conditions, the highest concentration of E2 (100ng/l nominal) caused ovipostion to continue at the same rate throughout the exposure period, relative to the pre-exposure period, while oviposition in the control and E2 groups declined, due to the cooler temperature and shorter photoperiod. This is not 'superfeminisation', as described by Oehlmann et al. (2006), but a perpetuation of the summer oviposition rate into autumnal conditions. Statistically, these are indistinguishable unless using a comparison between the reproductive performance of each replicate group in the baseline and exposure periods. This method was first used by Harries et al. (2000) when assessing the reproductive effects of 4-nonyl phenol in the pair breeding fathead minnow, *Pimephales promelas*. The method is now used in USEPA standard fish fecundity test protocols and in OECD test protocols because it accounts for the (considerable) between group variation in fecundity and separates the effects due of a chemical challenge from those inherent in each of the replicates.

5. Preliminary studies on the effects of E2 on *B. tentaculata*

5.1 Objectives

Because of the difficulty of maintaining *V. viviparus* in the laboratory, preliminary attempts were made to explore the suitability of another prosobranch, *B. tentaculata*, both in the mesocosm and in the laboratory. The advantages of *B.tentaculata* are that it is smaller, has separate sexes and is an egg-layer. It is found widely across the UK and Europe. Success in rearing *B. tentaculata* in the laboratory was limited because of its relatively specialised dietary requirements. As in previous experiments, *B. tentaculata* were sourced from the wild because there are no laboratory bred strains available. The objective was to make a preliminary assessment of *B. tentaculata*'s responses to E2, in the mesocosm and in the laboratory.

5.2 The exposure of B.tentaculata to E2 in an outdoor mesocosm

5.2.1 Methods

The snails were deployed in groups of 16, in six replicate cages, in each of the RW, LE2 and HE2 tanks. This experiment was run concurrently with experiment 2. The *B. tentaculata* were collected from the River Chelmer in Essex, immediately upstream of the mesocosm intake. They were fed *ad libidum* on pelleted fish food and organic carrot. The numbers of eggs laid were recorded every two weeks. At the end of the experiment all the surviving snails were sacrificed and their gender determined using a low-power microscope (x4 magnification). Any obvious parasite infections were noted.

5.2.2 Results

Survival

This small snail was difficult to contain in the mesh cages in the mesocosm tanks. Approximately 26% of the test snails escaped during the exposure. Therefore, the mortality rate in Figure 81 constitutes only the known deaths. There were no significant differences in the mortality rates between the groups.

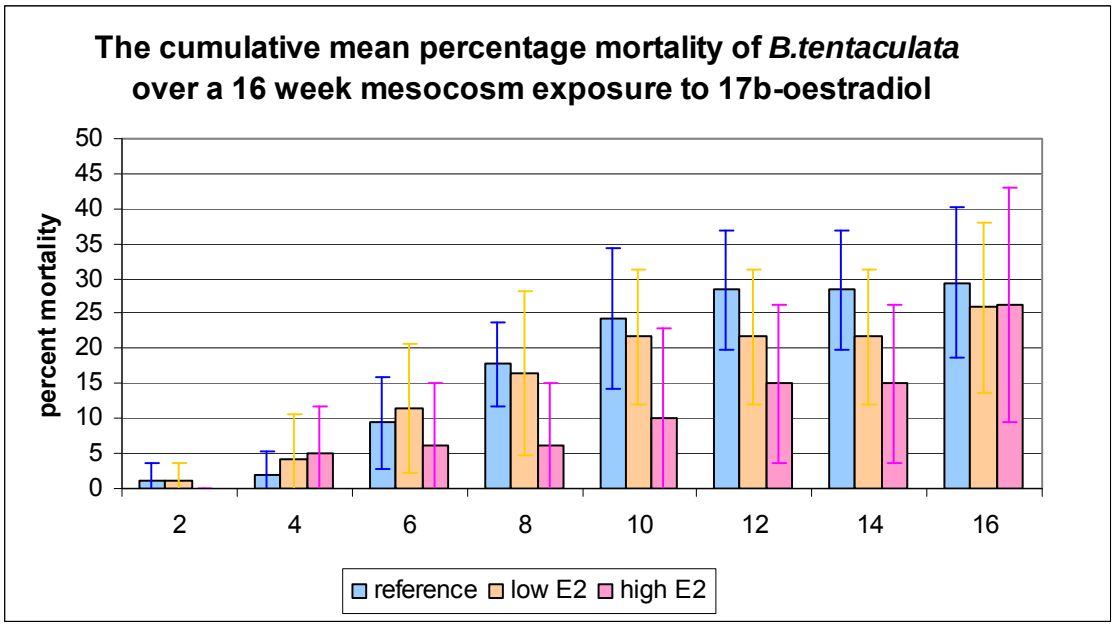


Figure 81. Cumulative percentage mortality of *B. tentaculata* over a 16-week mesocosm exposure to E2.

Growth

The population of snails deployed at the start of the exposure were approximately 8mm along their longest axis and approximately 0.1g in weight. There were no significant differences in length or weight between the control and exposure groups (P >0.05, Kruskal-Wallis test). The snails grew well over the 16-week exposure period. The RW group increased in length by approximately 15% and in weight by approximately 40% (Figure 80). Both of the groups exposed to E2 grew slightly more than this, the LE2 group increased in weight by over 50% and in length by over 20%. None of these differences were statistically significant (P >0.05, ANOVA).

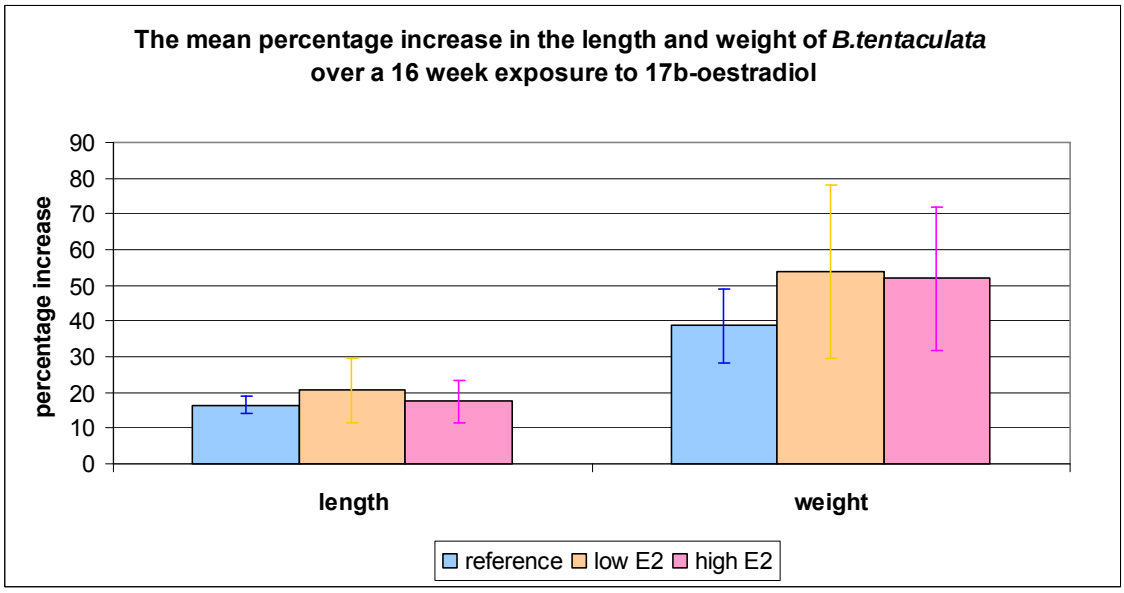


Figure 80. Mean percentage increase in length along the longest axis and weight of *B. tentaculata* over a 16-week mesocosm exposure to E2.

Reproduction

Reproduction was highly variable in each treatment group. On every sampling occasion the HE2 groups produced the most eggs per adult (assuming an equal sex ratio), but this was only statistically different from the RW treatment at week 14 (Figure 82).

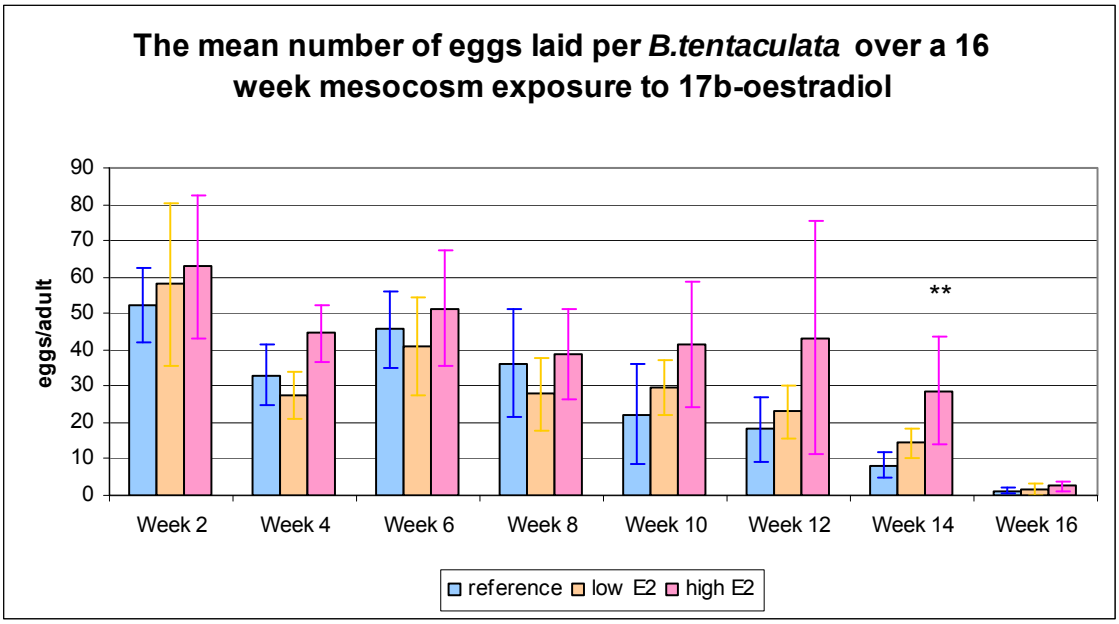


Figure 82. Mean number of eggs laid by surviving adult *B. tentaculata* over a 16 week mesocosm exposure to E2

Parasitism

When the snails were dissected at the end of the experiment it was found that in some replicates the remaining snails were either entirely female (one replicate in each of the lower and higher exposure tanks) or effectively incompetent (i.e. composed of males and parasitized females; one replicate in the higher exposure tank). These replicates were removed from the analysis.

5.2.3 Discussion

Reproductive output was highly variable, which may be due, in part, to the differing proportion of males and females in each replicate. This variable could not be controlled because of the large number of dead and escaped individuals. In the first half of the experiment, reproductive output was similar across treatments. In the second half of the experiment, reproductive output in the RW snails declined, as expected, but snails exposed to E2 continued to produce eggs at a higher rate. By week 14 there was a significant difference in the reproduction rate of RW and HE2 snails and an apparent dose-dependent response. Despite the difficulties caused by the loss of test organisms and the incidence of parasitism, this result indicates that exposure to E2 has an effect on reproduction in *B.tentaculata.*

5.3 The laboratory exposure of *B. tentaculata* to E2 during a 'simulated spring and summer'

5.3.1 Objective

The objective of this experiment was to expose *B. tentaculata* to E2 under controlled laboratory conditions and to confirm the effects, on egg productivity, that had been observed in the mesocosm. A high level of variability was encountered in the mesocosm experiment, so the snails were exposed in male and female pairs, allowing the variation at the level of individual females to be recorded. The snails were exposed in environmental conditions that were as similar as possible to those experienced during natural seasonal changes in temperature and day length. This was to allow for the seasonal effects on reproduction that were observed in all snail species studied. Consequently, the exposure was planned to begin in the early spring and to expose pairs of snails in controlled temperature and photoperiod conditions that reproduced a 'real-time' spring to autumn period of reproductive activity in wild snails. The hypothesis was that the rate of egg production in female *B. tentaculata* would be increased in snails exposed to E2, relative to the controls, during periods outside the peak of productivity (i.e. during a simulated spring and/or autumn), but not during the period of maximum reproduction (i.e. a simulated summer).

5.3.2 Methods

Experimental set-up

The experiment was performed in an air-conditioned room with insulated walls. The test vessels were placed on shelves lit by two Phillips 'Fluotone' strip bulbs. These emitted cool white light at approximately 3000 lux at the shelf surface. The photoperiod was automatically controlled. At the start of the experiment the photoperiod was 12 hours light and 12 hours dark, with lights switched on at 6.15am. As the natural season progressed, the photoperiod was adjusted in 'real-time', using sunrise and sunset times. The room was programmed to hold an initial temperature of 10^{o}C, commensurate with outdoor water temperatures in Manchester at the spring equinox when the exposure was planned to begin. The temperature was raised gradually to reach 20^{o}C by July (Figure 84).

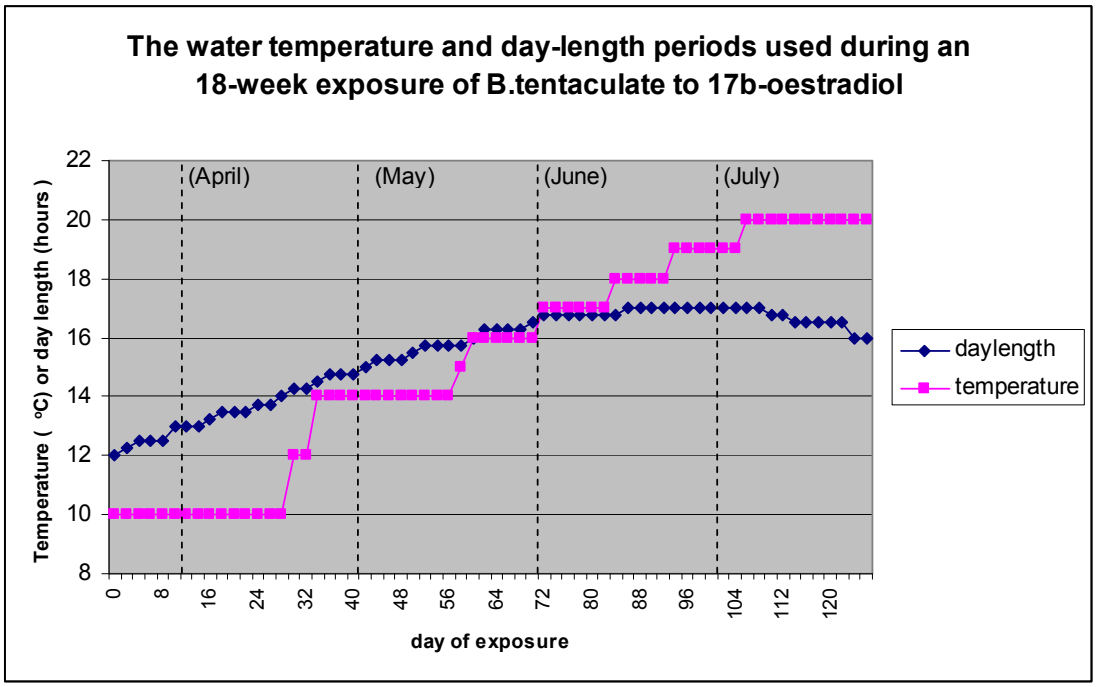


Figure 84. The water temperature and photoperiods employed during the 18-week exposure of *B. tentaculata* to E2.

The medium used in the experiment was Artificial Pond Water (APW) made using the recipe given for the *P.corneus* culture experiments. The water used to make the medium was filtered through carbon filters and reverse osmosis and allowed to equilibrate to the appropriate temperature for 24 hours prior to the addition of the salts. All of the media were replaced at 48 hour intervals. Following each medium change, the temperature, pH, dissolved oxygen and conductivity of the discarded water, from each control beaker, was measured. The samples were then pooled and the hardness of the discarded medium was measured by titration against EDTA in the presence of excess ammonia.

A stock solution of 100mg/l E2 in high purity methanol was prepared. The stock solution was stored, for the duration of the experiment, at 4 (± 2) ^{o}C in the dark. A 1mg/l solution of E2 in methanol was made and replaced fortnightly. To prepare the test medium, a 1.2ml aliquot of this was added to a 5l glass bottle and spread over the internal surfaces by tipping and revolving the bottle. The solvent was then allowed to evaporate. Finally, 120mls of reverse osmosis water was added to the bottle and mixed to make a 10μg/l solution of E2. Aliquots of 0.1, 1.0 or 10mls of this solution were then added to 1l of medium to make the nominal test concentrations of 1, 10 or 100ng/l E2, respectively. This was sufficient medium for use in five replicate beakers.

Test organisms

All the snails were obtained from 'Sciento' Biological Supply House, Manchester, having originally been collected from the inlet to a reservoir. They had been collected as they emerged from aestivation during late February and early March. Egg laying begins immediately on emergence from aestivation.

Before the start of the experiment, snails were drawn from the stock tank and individuals were weighed, measured along their longest axis and the sex of each animal was determined. No *B. tentaculata* less than 8mm long were used in the experiment to ensure that all the animals were sufficiently mature to lay egg masses reliably. The snails were fed a concentrated monoculture of *C.vulgaris*, which

amounted to 0.3mg of total organic carbon (TOC)/snail/day, this was delivered in a 2ml aliquot to each beaker. The *C.vulgaris* was cultured in the laboratory from stock supplies bought from the Culture Collection of Algae and Protozoa (CCAP), Dunstaffnage Marine Laboratory, Oban. The stocks were grown under sterile conditions in 2l volumes of Bold's Basal Medium 1.

Once separated into separate sexes the snails were randomly assigned (in male/female pairs) to consecutively numbered 250ml glass beakers that contained 200 ± 10ml of medium. The beakers were then randomly assigned to treatments and the treatments randomly assigned to their shelf positions, in groups of five beakers.

Sampling

The working volume in the exposure vessels was not sufficient to allow aliquots to be taken for chemical analysis, between media changes, without significantly affecting the volume of medium available to the snails. Consequently, five additional beakers, for the control and each concentration of E2, were maintained under the same conditions and with the same number of snails. The performance of these snails was not recorded, but the medium in these vessels was periodically taken for analysis, with the contents of all five 200 ml beakers making up the required 1l sample volume. These samples were taken for chemical analysis at six-week intervals. On the first occasion, the medium was sampled immediately post-dosing, replaced and then sampled after 24 hours, it was again replaced and then sampled after 48 hours. On subsequent occasions samples were only taken 24 hours after the medium had been changed.

At each media change (every 48 hours) the number of egg masses and eggs laid in each beaker were counted by eye (holding the beaker against a bright light). It was not possible to remove the egg masses without damaging the eggs and so no hatching studies were undertaken. All the snails were monitored for activity, every 48 hours, during the exposure. Individuals that were quiescent over 5 consecutive days were examined and a needle was carefully inserted under the edge of the opercula to assess the response of the snail. Live animals rapidly withdraw the opercula further into the aperture, whereas dead animals could easily be entirely removed from the shell. In this manner, the gender of all snails that died during the test could be established before decomposition occurred. When one member of a pair died the other snail was immediately sacrificed. At the end of the experiment, all the remaining snails were weighed, measured and their gender determined.

5.3.3 Results

Chemical analysis

The results of the chemical analysis are presented in Table 113. In the first sample the recovery of E2 from the freshly spiked medium was satisfactory (between 78.4 and 86.4% of nominal). This demonstrates that the preparation of the E2 stock solutions and the dosing procedure was adequate. As expected, the degradation rate of E2 over 48 hours was appreciable, with between 49.3 and 56.8% of the nominal concentration remaining after 24 hours and between 34.2 and 40.9% remaining after 48 hours. No E2 was recorded in the fresh media (although the LOD was rather high), but it was present in increasing amounts at 24 and 48 hours. This suggests that the females may have been contributing to the levels of E2 in the beakers. Similar E2 values were seen, on the second sampling occasion (12 weeks), in the controls and the nominal concentrations of 1 and 10 ng/l. By this sampling point, most of the E2 was being lost by 24 hours, possibly because of an adapted bacterial population and the increasingly

warm conditions. Only the highest nominal concentration (100ng/l) is distinguishable from the other treatments, with a measured concentration of 4.09ng/l. The same is true on the third sampling occasion (18 weeks), where the highest E2 concentration is 19.7ng/l, giving a mean value of 26.3ng/l (SD = 26.1) for the three 24 hour sampling points. At this sampling point the E2 concentrations for the control and other treatments were, again, indistinguishable from one another.

Table 113. The results of the E2 and E1 analysis (expressed in ng/l) performed during an 18 week exposure of *B. tentaculata.*

		control	1 ng/l nominal	10 ng/l nominal	100 ng/l Nominal
First sampling occasion (6 weeks)					
0 hours	*17β-estradiol*	<0.15	0.784	8.57	86.4
	Oestrone	<0.5	<0.5	<0.5	<0.5
24 hours	*17β-estradiol*	0.169	0.493	5.68	55.1
	Oestrone	<0.5	<0.5	0.578	7.22
48 hours	*17β-estradiol*	0.257	0.342	4.09	39.2
	Oestrone	<0.5	<0.5	<0.5	5.72
Second sampling occasion (12 weeks)		0.271	0.189	0.355	4.09
24 hours	*17β-estradiol*	<0.5	<0.5	<0.5	<0.5
	Oestrone				
Third sampling occasion (18 weeks)					
24 hours	*17β-estradiol*	1.18	1.91	0.964	19.7
	Oestrone	ND	1.46	3.84	30.7

The mean pH of the freshly prepared medium was 7.49 (± 0.17 SD). The mean dissolved oxygen levels ranged from 59 to 76.6%. These are adequate levels for respiration in gill dependent organisms when it is considered that these measurements were taken in the discarded medium. The mean (± SD) dissolved oxygen level of the new medium over the course of the exposure was 101.6% (± 3.4). There was an increase in conductivity over the course of the exposure (from 780 us/cm to ~1000 us/cm). The mean (± SD) hardness of samples of fresh medium, taken over the duration of the experiment, was 424mg/l $CaCO_3$ equivalents (± 44). The mean hardness of the discarded medium ranged between 400 and 434mg/l $CaCO_3$ (equivalents).

Table 114 shows the mean, minimum and maximum temperatures measured in the discarded medium following each medium change.

Table 114. The mean, minimum and maximum temperatures experienced by the exposure vessels for each fortnight of the experiment.

	Temperature (°C)		
Exposure Period (Fortnightly)	**Mean**	**Minimum**	**Maximum**
1. *21st March to 4th April inclusive*	10.07	10	10.5
2. *5th April to 18th April inclusive*	10.00	10	10
3. *19th April to 2nd May inclusive*	13.36	12	14
4. *3rd May to 16th May inclusive*	14.07	14	14.5
5. *17th May to 31st May inclusive*	15.86	15	16
6. *1st June to 13th June inclusive*	16.93	16.5	17.5
7. *14th June to 27th June inclusive*	18.29	17.5	18.5
8. *28th June to 11th July inclusive*	19.58	19	20
9 *12th July to 25th July inclusive*	20.00	20	20

Effects of E2 exposure on *B.tentaculata*

Mortality

The overall mortality in the experiment was high, reaching 26% in the control group. In all groups there was slightly higher mortality amongst the males than the females (Figure 85). Significantly more of the total population in the 1ng/l E2 group died than in the control group ($P < 0.05$, ANOVA), but there was no obvious trend in mortality rates with increasing E2 dose. There were no significant differences between the control group and any treatment group for male or female mortality rates.

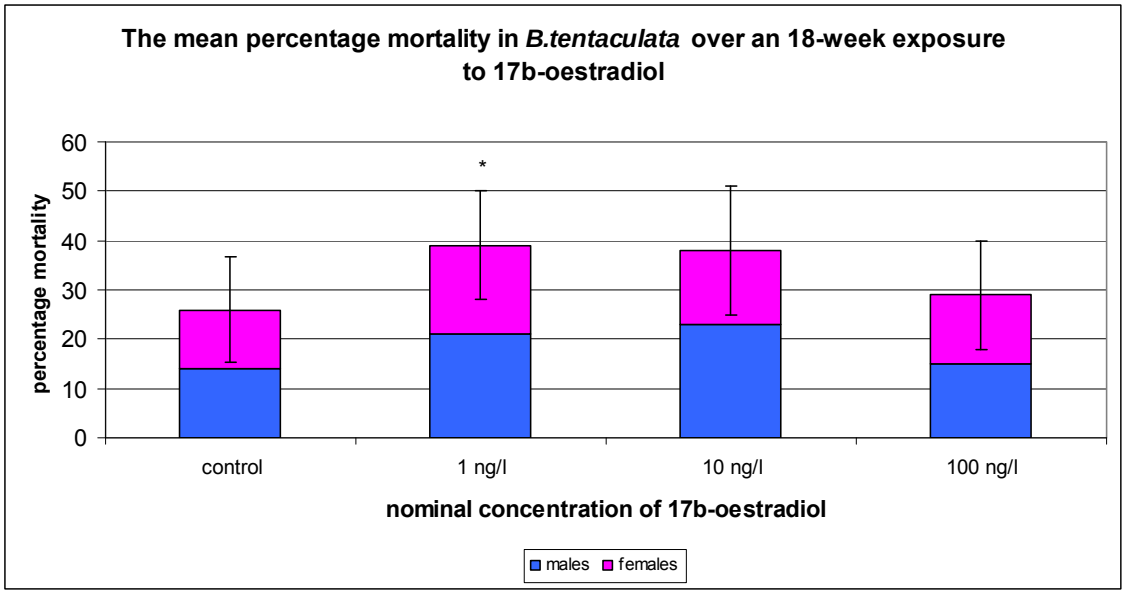


Figure 85. The percentage mortality of male and female *B. tentaculata* over an 18-week exposure to E2.

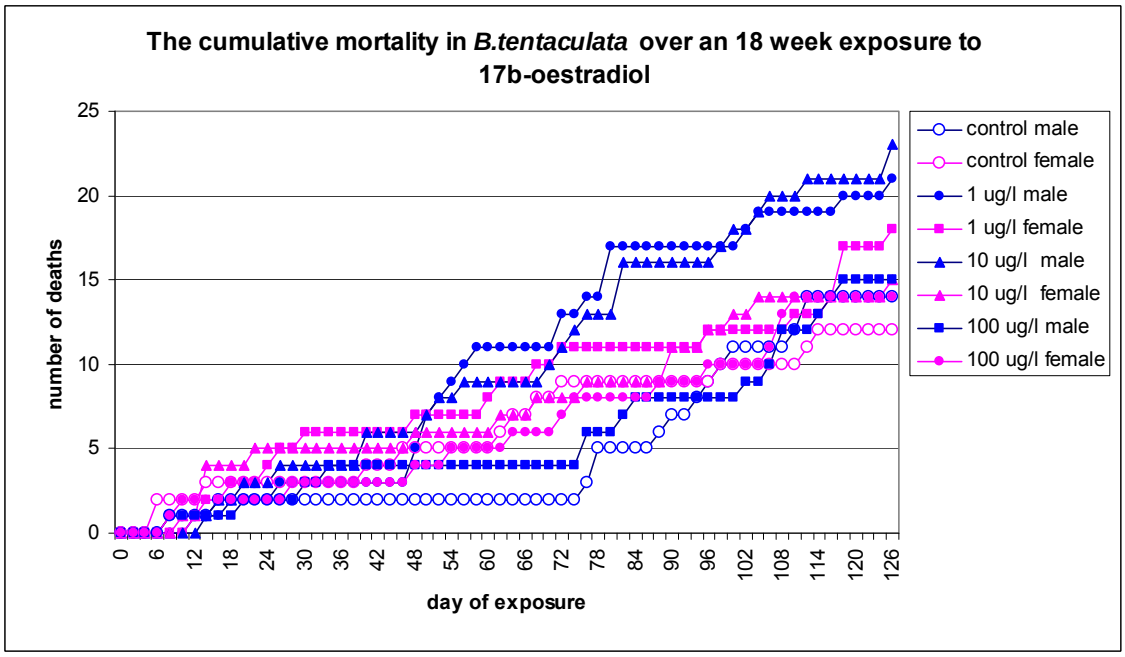


Figure 86. The cumulative mortality of *B. tentaculata* over an 18-week exposure to E2.

Growth

At the start of the experiment, the mean length, along the longest axis, and the mean weight of the control and treatment groups were very similar, approximately 11mm and 0.22mg. There were no significant differences between the groups (P >0.05, ANOVA for length analysis, Kruskal-Wallis for weight analysis). Length measurements were abandoned after ensuring all the test snails were >8mm long, as there was a tendency for the spire to be rubbed off and this leads to false figures for body size. The length of the aperture, along the longest axis, was measured instead (Table 119).

Table 119. The mean length of the aperture and the mean weight of test organisms at the start, of those that died or were sacrificed during, and at the end, of an 18-week exposure of *B. tentaculata* to E2 (all concentrations are nominal).

	Aperture length (mm)			Weight (g)		
	At start	Died during	At end	At start	Sacrificed During	At end
Control (SD)	5.37 (0.34)	5.42 (0.32)	5.40 (0.36)	0.21 (0.04)	0.23 (0.03)	0.22 (0.05)
1 ng/l (SD)	5.40 (0.37)	5.51 (0.40)	5.49 (0.38)	0.22 (0.05)	0.22 (0.04)	0.25 (0.06)
10 ng/l (SD)	5.39 (0.37)	5.43 (0.43)	5.47 (0.34)	0.22 (0.05)	0.22 (0.05)	0.23 (0.06)
100 ng/l (SD)	5.40 (0.34)	5.56 (0.30)	5.36 (0.34)	0.22 (0.04)	0.22 (0.04)	0.22 (0.04)

Growth over the whole exposure period (18 weeks) was barely perceptible. Aperture size in the control group increased by 0.56% and by 1.67% and 1.5% in the two lowest E2 concentrations. Aperture size in the 100ng/l (nominal) group appears to shrink because larger animals had a higher mortality rate. The weights of all the groups did not alter, indicating that the snails did not lose condition over the course of the exposure.

Reproduction

Of the 200 pairs in the experiment, only 21 produced eggs. Figure 88a shows the mean number of eggs produced per reproducing pair over the 18 week exposure. There were no significant differences in egg productivity across the groups. However, Figure 88b shows the performance, in detail, of each of the pairs exposed to 100ng/l (nominal) E2. Of the seven breeding pairs in this group, four had a similar mean total egg productivity to that observed in the control group, but the remaining 3 pairs had productivity that was 2-3 times above the expected level.

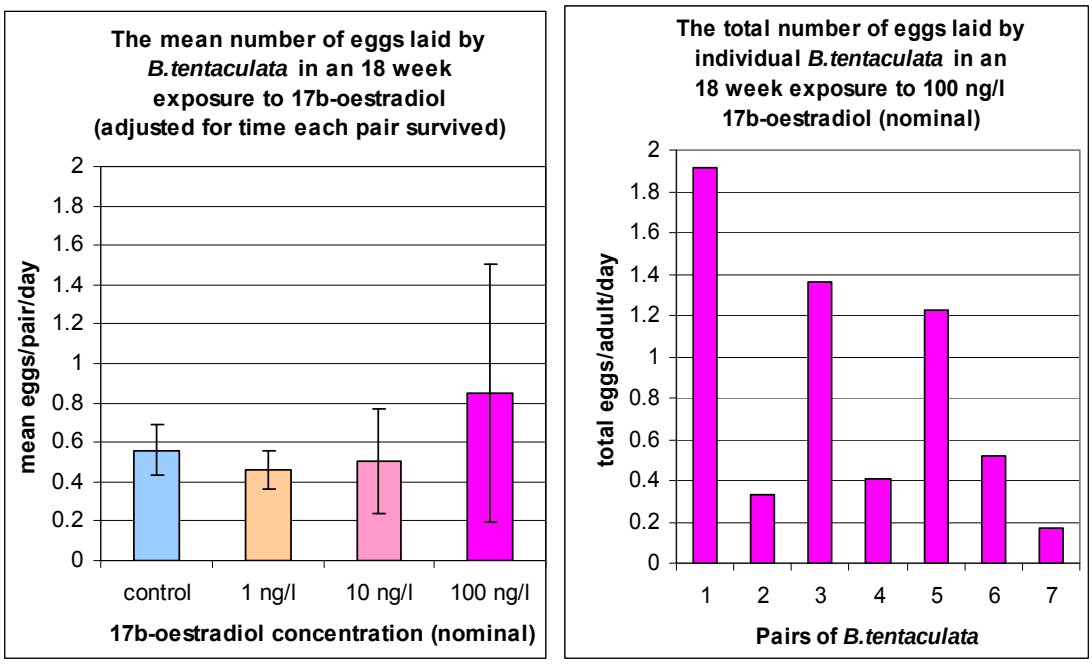


Figure 88. Mean number of eggs laid/pair/day over an 18-week exposure of *B. tentaculata* to E2 (a) and the total number of eggs laid by individual pairs exposed to 100 ng/l E2 for this period (b).

Figure 89 shows the mean number of eggs laid per surviving pair when the data is plotted as a time course. This shows that the cumulative increase in the mean productivity of the 100ng/l (nominal) E2 group is elevated, compared to the controls, both in the early stages and towards the end of the experiment.

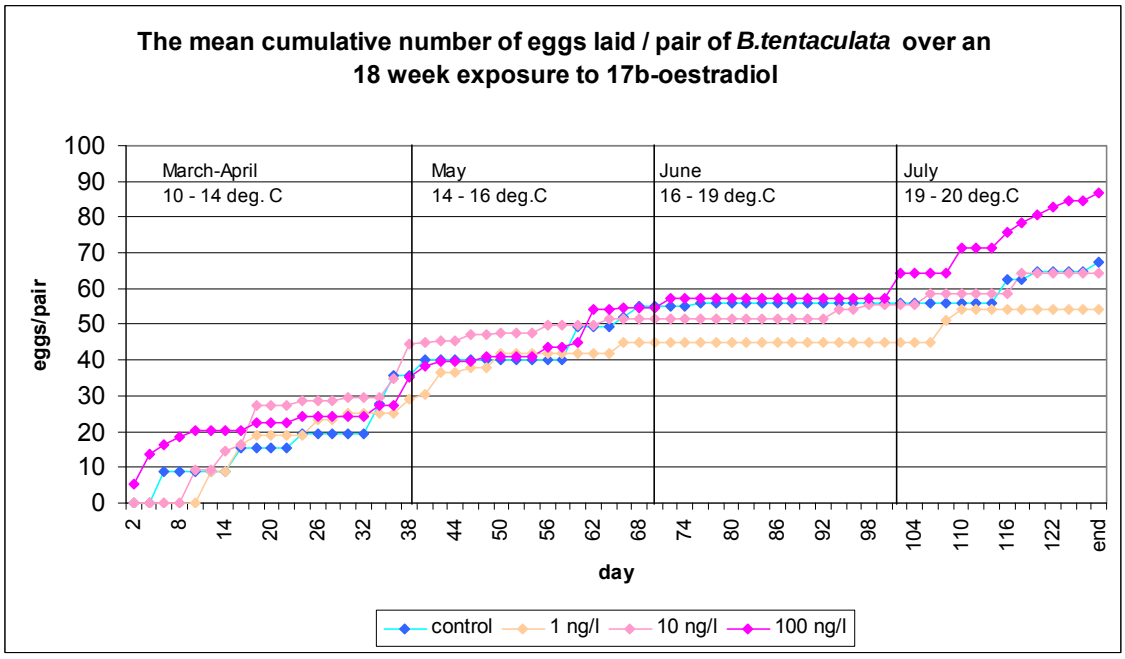


Figure 89. Mean cumulative number of eggs laid per surviving egg-laying pair of *B. tentaculata* over the course of an 18-week exposure to E2 (For clarity only the control group standard deviations are shown).

Parasitism

Parasites were found in 35% of the animals dissected during the experiment and in 49% of the snails remaining at the end of the experiment. Parasites were rarely seen in dead snails,

5.3.4 Discussion

It was difficult to maintain the concentrations of E2 in the test vessels, possibly due to their small volumes. However, the 100ng/l nominal exposure group could be regarded as distinct from the other groups because the concentrations of E2 measured in this treatment were, on average, 10.5 times higher, on each sampling occasion, than the concentrations measured in any other group. There was no equivalent difference between the other groups, except on the first sampling occasion, so all the other groups (including the controls) can only be described as being exposed to low levels of E2.

The dissolved oxygen concentrations in the discarded media were lower than expected and on occasion were as low as 32%. It is not clear why this occurred, but it may have contributed to the high levels of mortality observed. The test vessels held only 200 ml of media and that may have been an insufficient volume to maintain a level of water quality acceptable to this species. Alternatively, the high mortality rate may have been due to the very high levels of parasitism recorded. The first parasitized animals were not seen until May, when the water temperature had reached 14^{o}C. Given the increasing incidence of parasites, without a transfer vector present, it is likely that around 50% of the test organisms were parasitized from the outset. It is also possible that all the animals were parasitized, but that some infections were not sufficiently heavy, or did not reach a late enough stage, to be visible on microscopic inspection.

Of the 200 pairs, only 21 produced egg masses during their 18-week exposure. This low egg production could have been due to: incompatibility of the pairs (a drawback of pair-breeding studies, where a choice of mate is necessarily denied to the individuals); unsuitability of the test medium or housing of the snails; and/or high levels of parasitism in the population. The higher reproduction rate in the groups exposed to 100ng/l E2 was due, in part, to there being more egg-laying pairs in this group than any other (5 control pairs laid eggs, 4 in 1ng/l nominal, 5 in 10ng/l nominal and 7 in 100ng/l nominal). An analysis of mean reproductive output per day from individual pairs, shows that some pairs produced a greater number of eggs (>2 – 3 times the numbers produced by any other pairs). The increased egg-laying in the 100ng/l E2 group, over the control group, may be an effect of the steroid, but this is not clear because the numbers of egg laying pairs was too low overall. The times when egg laying in this group increased coincided with the ‘spring’ and the ‘onset of autumn’. This supports the hypothesis that E2 exposure increases reproduction, above the control rate, outside of the period of peak productivity.

6. General Discussion

The results of this research project suggest that the seasonal reproductive cycle in two native mollusc species, *V. viviparus and P. corneus*, is altered by exposure to E2 under semi-field (mesocosm) conditions. In *P. corneus*, these findings were corroborated in laboratory experiments, where E2 exposure appeared to prolong egg laying under short day and low temperature conditions, but had no effect on snails exposed to the same E2 concentrations under longer days and higher temperatures. There were indications that early gonadal development in F1 snails is more sensitive to E2 and other chemicals than in the F0 generation. E2 exposure during development resulted in: poorly differentiated gonads; intersexuality; changes in the sex ratio of surviving viviparid offspring (although the numbers collected were low); and disruption of gametogenesis in planorbid offspring (Sertoli cell disruption and a decrease in the activity of the vitellogenic area). Some of these effects (particularly on the oocytes) persisted even after a year of depuration. In viviparid offspring, exposure to EDCs during their development, increased the likelihood of parasitism. This correlation was found by histological analysis of tissue sections, highlighting the importance of the use of histological methods.

The prolongation of egg laying in *P. corneus*, increased embryo production in *V. viviparus* and the effects on the F1 generation in both species are potentially important. It has been proposed that exogenous sources of vertebrate steroid hormones, or other steroid receptor agonists, can contribute to an increase in reproductive activity (Oehlmann et al. 2000, 2006) and that this may be a form of endocrine disruption (Oehlmann et al. 2007, 2008). In *M. cornuarietis* exposed to Bisphenol A, this effect, increased reproduction, was only apparent when the exposure was made at relatively cool temperatures and during phases of reduced reproduction. The effect was 'masked' when the snails were exposed to more tropical temperatures. The results support this observation, that EDC effects interact with season/temperature induced changes in reproduction.

The effects of E2 and mixtures of oestrogenic chemicals on the F1 generation included a high mortality rate in the offspring. In surviving snails, histopathology of the gonads revealed evidence of inhibition of sexual development in one or both sexes. The mechanisms for these effects are not known. Molluscs are known to have estrogen-like receptors, but it is not known whether steroids can modulate a form of endocrine control through these receptors, as they do in vertebrates. Further work in this area is discussed in an additional report (Environment Agency 2009).

Limitations of the research

The conclusions drawn from this study are limited by several factors, the most important of which were the levels of parasitism observed, the variability in reproductive output between groups of snails and interpretation of the actual concentrations of chemicals that the snails were exposed to.

In the mesocosm experiments, it is important to mention that there were no true control tanks. In all the experiments the diluent was river water that already contained variable amounts of E2 and other chemicals. The results from experiment 1 clearly show that both estradiol and oestrone were present in the river water control tanks on all occasions. The analytical results from two laboratories corroborated one another and samples taken from the river water inlet (subsequently) have verified that these

steroids are intermittently present in the river at the inlet point and that this was not due to inadvertent contamination of the tanks.

The measured concentrations of the dosed estrogens in the treatment tanks were highly variable. The variation was such that in experiments 2 and 3 no statistically significant differences in concentrations were found between the low and high doses, over the course of the experiment. This was possibly due to macro-algal growth in the tanks and the chemical composition of the diluent. A change was made from using river water stripped over activated carbon filters in experiment 1, to unstripped river water for experiments 2 and 3. This was because the *V. viviparus* snails lost weight in experiment 1, as there was less organic matter and, therefore, natural food available in the stripped river water. There was also microbial growth in the dosing reservoirs, despite the use of chlorinated tap water as a diluent. However, spot samples showed that there were differences in the concentrations of E2 in the LE2 and HE2 treatments, even though the measured values decreased over the duration of the experiments. Experiment 3 was conducted at the same time as experiment 2 and so there was no opportunity to learn from experience. Except for the steroids, the only differences between the LM and HM tanks were for NP, which was consistently measured at a higher concentration in the HM tank than the LM tank. Again, spot samples showed that there were differences in the amounts of some of the dosed chemicals, between the LM and HM tanks. The biological results also suggest that the HE2 and HM tanks contained higher concentrations of dosed chemicals than the LE2 and LM tanks.

In the laboratory, E2 was present in the control tanks indicating they may have been contaminated with E2. The mean reported level of E2 in the control tank, in the *P.corneus* exposures, was below the MRV of 0.3ng/l. The rest of the control samples were within the range that has been reported from fish experiments, in semi-static or slow flowing exposures, where both males and females are present and females can release E2 into the water (Lower et al. 2004; Scott & Ellis, 2007; Thorpe et al. 2009). Snails are known to produce E2 (Jane & Porte, 2007) so small traces of E2 in the control tanks, on some occasions, should not be unexpected. However, in the *B.tentaculata* experiment the E2 concentrations in the control and the 1 and 10ng/l (nominal) concentration vessels became indistinguishable. This was probably due to steroid production by the snails combined with a high level of microbial degradation in the small volumes of water in the containers.
The high levels of parasitism among the snails added to the difficulty of interpreting the biological results, particularly any effects on reproduction (which trematode parasites are known to inhibit). Parasitic infestation was not addressed in this project. Parasite infection was not observed until the end of the laboratory study into the effect of E2 exposure on *P.corneus* and only a small proportion of the snails were affected (6.5%). Parasites were then found by histological analysis of the snails in the mesocosm experiments. Finally, high levels of infestation were encountered in the *B.tentaculata* experiments. The only solution to this issue will be to establish stable, parasite-free, laboratory populations of the test species. The long life cycles of these species mean that it will take a minimum of two years to achieve this.

Parasite infections were, at least in part, responsible for the variation in reproductive output between groups of wild caught snails. Notwithstanding this, fecundity is inherently variable, even in laboratory bred populations of fish. As is common practice in fish toxicological studies, using fathead minnows and zebrafish, variability was minimised by selecting snails, (a) prior to testing chemicals, to remove underperforming groups and, (b) after testing (in both mesocosms and lab experiments) to correct for parasitised individuals. This is necessary because there is evidence that, in *P. corneus*, heavily parasitised snails do not breed. In *B. tentaculata*, there is some indication that some parasitised individuals do breed. On balance, it was better to remove the data from parasitised snails because it *may* influence their breeding.

Variability in reproductive output between groups, and pairs, of snails is certainly a problem with all snail studies. Forbes et al. (2007b) identified this as a major factor in experiments. After removing parasitised snails, the standard deviations for egg production were, in most cases, similar to those reported by Forbes et al. (10 to 45% and averaging 25%).

7. Final conclusions

The main aim of this project was to explore whether native mollusc species could be developed as test organisms for use in regulatory tests for endocrine disrupting chemicals. To meet this aim it was necessary to identify native species that could be cultured in the laboratory and then to optimise the culture conditions, so that their sensitivity to endocrine disrupting chemicals (mainly 17β-estradiol) could be assessed. Prior to the laboratory experiments, a number of adult exposure experiments and two whole life cycle exposure experiments (17β-estradiol and a mixture of EDCs) were carried out in outdoor mesocosms. This allowed the responses of the snails to EDCs to be assessed and likely candidate snail species to be identified.

In this report the effects of E2 on adult survival, growth and reproduction of *P. corneus*, *V. viviparus* and *B. tentaculata* in outdoor mesocosms are described. The effect of a mixture of EDCs on adult survival, growth and reproduction of *P. corneus* and *V. viviparus* and the effects of E2 and EDCs on development and reproduction in the F1 generation of these two species are reported. *P. corneus* snails were exposed to E2 under two different temperature regimes (summer and autumn) to explore how seasonal variation in reproduction might interact with chemical effects on reproduction. *B. tentaculata* were also exposed to E2 under laboratory conditions. The feasibility of culturing all of these species, in the laboratory, has been studied. *P. corneus* (a pulmonate hermaphrodite egg layer) showed the most promise. Other pulmonate species can be kept under the conditions developed for this species. Establishing laboratory populations of prosobranch species (*V. viviparus* and *B. tentaculata*) proved more difficult and it was not possible to establish stable, long term populations during this project.

In all the species tested, reproduction was affected by exposure to E2 in outdoor mesocosms. *P. corneus* and *B. tentaculata* were also affected by E2 exposure under laboratory conditions. The effects on adult reproduction can be inhibitory or stimulatory, depending on chemical concentration, environmental conditions (seasonality) and chemical toxicity. Identifying the concentrations of estrogenic chemicals that had effects on mollusc reproduction in the mesocosms was confounded by the real world aspects of this approach e.g. bacterial degradation, sorption to sediments, algal growth and the presence of steroids in the river water used as diluent.

The reproductive output of wild caught snails was highly variable between individuals and groups. Regulatory tests use laboratory strains, which are much more uniform in their responses to chemical challenges. The source of the variability observed is likely to be due to a combination of factors, including age, maturity, health and genetics. Indeed, histological analysis has shown that parasitism is common in many species of mollusc collected from the wild. Many parasites can, directly or indirectly, affect reproduction and some of the variation in reproductive output observed in groups of snails may have been due to the frequency and intensity of parasitism. Many of these factors could be controlled by using established laboratory strains and the availability of such strains would improve the reliability of tests. For this reason, the establishment of laboratory strains should be a priority.

Histological analysis of the gonads has shown that EDCs can disrupt both the male and female components of gametogenesis. However, in *P. corneus* and *V. viviparus*, disruption of the vitellogenic area of the gonad (i.e. oocytes) is far more persistent than any short-term activational effects on spermatogenesis. Early stages in the life cycle appear to be more susceptible to the effects of estrogenic chemicals, than adults. For example, F1 snails that had been exposed during their development displayed both morphological effects on the accessory reproductive organs and disrupted gametogenesis leading to a failure to reproduce. Therefore, further research on the

developmental effects of EDCs on native molluscs should be a priority. These results indicate that developmental exposure to EDCs may increase the likelihood of parasitic infection, possibly due to suppression of the immune system.

Finally, when developing mollusc tests, it will be important to consider the effects of temperature and seasonality on reproduction. This data indicates that chemical effects on reproduction are most likely to be observed when the rate of reproduction is changing (i.e. in spring or autumn conditions). This was demonstrated in the mesocosm studies of exposure to E2 and in the laboratory exposures of *P.corneus*. It has also been reported in previous studies with treated sewage effluent (Clarke *et al.,* 2009). Experimental protocols that depend on changing environmental conditions may be difficult to implement. It is possible that the strong dependence of reproduction on seasonal cues may become less pronounced in laboratory bred animals.

Overall, there is evidence that reproduction and early development in a number of species of native freshwater snail are affected by chemicals and the life cycle studies suggest that this may have effects at the population level. Given the variability in responses found in wild caught snails, it is essential that laboratory strains are established, as the next step towards the development of reliable and useful tests that use native molluscs.

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