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17β-Oestradiol may prolong reproduction in seasonally breeding freshwater gastropod molluscs

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ABSTRACT

Whilst the effects of oestrogenic contaminants in the aquatic environment are well documented in fish, effects in invertebrate species has been subject to debate, possibly due to differences in experimental conditions (temperature, timing and duration of exposure) between studies. It has been suggested that molluscs are only susceptible to oestrogens in periods either following the main spawning or leading up to the maturation of gametes. To investigate this possibility, two temperate, seasonally reproducing gastropods (*Planorbarius corneus* and *Viviparus viviparus*) were exposed to two concentrations of 17β-oestradiol (E2; 10 ng/l and 100 ng/l nominal) in an outdoor mesocosm (subject to natural seasons). In addition, *P. corneus* was also exposed to E2 (1, 10 and 100 ng/l) in the laboratory at temperatures and photoperiods to simulate summer and autumn.

In the mesocosm, both snail species produced similar numbers of eggs/embryos as reference groups in the summer, but the groups exposed to 10 ng E2/l (nominal) had significantly higher productivities after the onset of autumn, when entering their quiescent phase, whilst the snails exposed to a higher concentration (100 ng/l, nominal) had an increased rate of mortality, and did not experience increased reproduction. In the laboratory, the rate of egg laying in *P. corneus* was unaffected in simulated summer (20 °C, 16 h photoperiod), but snails exposed to 10 and 100 ng/l (nominal) in simulated autumn (15 °C, 12 h photoperiod) showed a concentration-dependent inhibition of the natural decline in egg laying observed in the control snails. Overall, rather than an increase in reproductive rate, the response of this species was a perpetuation of summer reproductive rates into autumn. We conclude that exposure to E2 can affect reproduction in the freshwater gastropods studied, but in *P. corneus* at least, this is dependent on the seasonal conditions (temperature and photoperiod) at which exposures are made.

1. Introduction

The oestrogenic steroid compounds 17β-oestradiol (E2), oestrone (E1) and 17α-ethinylestradiol (EE2) are discharged to freshwater environments in treated sewage effluents, and are known to contribute to feminising effects in male fish, through their interaction with oestrogen receptors (Sumpter and Jobling, 1995; Rodgers-Gray et al., 2001; Jobling et al., 2002a,b; Katsu et al., 2007). Structural oestrogen receptor analogues also occur in several classes of molluscs (Thornton et al., 2001; Keay et al., 2006; Kajiwara et al., 2006), together with sex steroid hormones and their enzymes (Reis-Henriques et al., 1990; Croll and Wang, 2007), although the function of these receptors in reproduction remains elusive as there is currently no evidence to suggest that mollusc oestrogen receptors bind to sex steroids. However, studies in the scallops *Patinopecten yessoensis* and *P. magellanicus*, have indicated that oestrogens enhance (Kadam et al., 1991; Osada et al., 1998) or potentiate (Osada et al., 1992; Wang and Croll, 2006) spawning indirectly, probably by up or down regulating the serotonin receptor, which is known to be involved in regulating the spawning response in another bivalve mollusc, *Mytilus edulis* (Cubero-Leon et al., 2010). As the expression of this receptor varies with the stage of sexual development, bivalve molluscs may be susceptible to feminisation effects only in the quiescent period leading up to the maturation of the gametes (Mori, 1969; Osada et al., 1992; Cubero-Leon et al., 2010).

This theory may also apply to gastropod molluscs. Oehlmann et al. (2006) reported increased rates of oviposition, and enlarged female pallial accessory glands in the gastropod mollusc *Marisa cornuarietis* exposed to oestrogenic compounds (EE2 and Bisphenol A, a plasticiser compound known to be oestrogenic in fish; Sohoni

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et al., 2001) but only at 20–22 °C under a 12:12 h light/dark photoperiod (outside of spawning conditions), and not at 27 °C when the females were fully spawning. Confounding evidence was later provided by Forbes et al. (2007a,b), who could not confirm these 'superfeminisation' effects in *M. cornuarietis* exposed to Bisphenol A at either 22 or 25 °C. Interestingly, the populations of *M. cornuarietis* that were affected by chemical exposure exhibited an annual spawning season (Oehlmann et al., 2006), whereas those that were unaffected did not (Aufderheide et al., 2006; Forbes et al., 2007b). Consequently it is possible that there is a seasonal component to the effects seen that is difficult to reliably reproduce in this subtropical species. In order to address the possibility that oestrogens increase the rate of oviposition in gastropod molluscs only under certain seasonal conditions, we exposed two temperate, seasonally reproducing species of gastropod; the hermaphrodite pulmonate *Planorbarius corneus* and the ampullid gonochoristic prosobranch *Viviparus viviparus*, to E2 in an outdoor mesocosm system over a natural summer and autumn. Laboratory exposures of *P. corneus* were also performed under temperatures and photoperiods to simulate both summer and autumn. In this way, any transitory effects of E2 on oviposition rates could be more closely observed.

2. Methods and materials

2.1. Mesocosm exposures

P. corneus and *V. viviparus* were deployed into in three large (1 m^3) outdoor mesocosm enclosures continually fed with river water from the River Backwater at 51°74′N, 0°66′E. The flow-through rate was approximately 3 l/min and the retention time was 5–6 h. The snails were housed in cylindrical mesh cages 80 cm tall and 50 cm in diameter that were suspended in the water to a depth of approximately 60 cm. The *P. corneus* were supplied by 'Blades' Biological Supply (wild caught), and the *V. viviparus* were collected from a local site (51°53′N, 0°49′W). The snails were weighed and measured along their longest axis before being randomly allocated to the enclosures in groups of 9 (*P. corneus*) or 11–13 (*V. viviparus*) per cage. There were three (pseudo-) replicate cages per species per enclosure. The *P. corneus* were fed on pelleted fish food (TetraPond floating food sticks, Melle, Germany) and organically grown carrot slices *ad libitum*. The *V. viviparus* filtered the natural supply of algae in the river water.

Two of the enclosures were continuously dosed with E2 (CAS 50-28-2, >98% purity; Sigma, Poole) from a stock solution of 1 g/l in ethanol to attain nominal concentrations of 10 or 100 ng/l. The solvent carrier did not exceed 0.001 ul/l in the enclosures. On exiting the system, the river water from all three enclosures passed through an activated carbon filter to remove the steroid prior to being discharged. The exposure period ran for 16 weeks from early May until late August, spanning the peak of the annual reproductive cycle. The *P. corneus* egg masses were collected fortnightly from glass inserts placed into the cages to act as oviposition surfaces and the eggs were counted under a stereo-microscope. The juvenile *V. viviparus* were also collected from the cages fortnightly and counted. Weekly checks for adult mortality were made, and any dead snails were removed. The calculated number of offspring per adult was adjusted accordingly. At the end of the exposure, all the surviving snails were re-weighed and re-measured, and the adult female *V. viviparus* were narcotised in 5% $MgCl_2$ and dissected to remove all of the embryos in the brood pouch.

2.2. Laboratory exposures

Two separate semi-static exposures of *P. corneus* to E2 were performed, one at 20 °C with a photoperiod of 16/8 h L/D (simulated summer) and one at 15 °C with a photoperiod of 12/12 h L/D (simulated autumn). The light phase in both was approximately 4000 lux. Approximately 50% of the *P. corneus* were supplied by 'Sciento' and ~50% from Queensborough Tropical (both wild caught). These were intermixed before being randomly allocated in groups of 6 into plate glass tanks (15 cm wide × 30 cm long × 30 cm high) with a working volume of 9 l. The snails were fed on fish flake (King British, Sinclair, UK) at a rate of 0.25 g per snail per 48 h. There were 5 replicate groups per treatment in the summer study and four in the autumn study.

The test medium was an artificial pond water (APW; 294 g/l $CaCl_2 \cdot 2H_2O$, 123.25 g/l $MgSO_4 \cdot 7H_2O$, 64.75 g/l $NaHCO_3$ and 5.75 g/l KCl diluted with reverse osmosis carbon filtered water). Aeration was provided at 200 ml/min via an air filter and glass pipette. Six litres of media were replaced every 48 h with the snails being allowed to reside in the remaining 3 l. The faecal strings and any remaining food were removed at each medium change. Any adult snail mortalities were recorded, and the volume of water in the tanks was adjusted in order that the stock density remained constant. The temperature, pH and dissolved oxygen were measured in the waste medium from each control tank. The hardness of the medium was also measured fortnightly.

The snails were allowed to acclimate to the test conditions for 1–3 weeks. Following this, there was a 4-week period during which the baseline reproductive rate of each replicate group was recorded. The egg masses were collected every 48 h and the eggs were counted using a stereo-microscope, and the number of eggs per adult per 48-h period was calculated, adjusting for any adult mortalities observed. On completion of the baseline, the tanks were then randomly allocated to treatment. A short E2 degradation study was conducted to assess the linearity of steroid degradation in the test conditions (refer to Fig. SI-1). At each medium change, the replacement medium for the E2 treatments was then spiked from a 10 ug/l working solution of E2 in APW to give nominal E2 concentrations of 1, 10 and 100 ng/l in the exposure tanks (the working solution was prepared by drying a 1 ml aliquot of a 10 mg/l stock solution of E2; CAS 50-28-2, >98% purity; Sigma, Poole) in methanol and re-wetting into 1 l of APW). The exposure continued for 8 weeks following the same conditions as the baseline period. At the end of the exposures all the surviving snails were re-weighed and re-measured.

2.3. Analytical chemistry of water samples

In the mesocosm exposure, monthly 1 l samples were taken from each tank and preserved with $CuSO_4$. In the laboratory exposure, equally sized aliquots were taken from each tank at fortnightly intervals, and pooled into a 500 ml sample for each concentration, both before and after a partial medium change. All samples were passed through a C18 SPE column and the column dried completely by drawing through air. The columns were extracted with dichloromethane before being evaporated to a small volume and cleaned up using a gel permeation chromatography fractionation. The extract was then solvent exchanged to 95:5% (v/v) iso-hexane:propan-2-ol, cleaned up using normal phase chromatography (LC column) and then evaporated to incipient dryness and dissolved in aqueous methanol, before analysis using HPLC with a negative ion atmospheric photo-ionisation interface and MS time-of-flight detection (National Laboratory Service, Environment Agency).

2.4. Statistical methods

In the mesocosm study, the interpretation of data was principally by the assessment of trends in reproductive rates for *P. corneus*, but a comparison of the (pseudo-) replicate means was also