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Adult exposure to the synthetic hormone 17α-ethynylestradiol affects offspring of the gastropods *Nassarius burchardi* and *Nassarius jonasii*

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ABSTRACT

The aim of this study was to determine whether adult exposure to endocrine disrupting compounds affects offspring using trans-generational testing. Adult estuarine dwelling gastropods *Nassarius burchardi* and *Nassarius jonasii* were exposed to the synthetic estrogen 17α-ethynylestradiol (EE2) to determine the effects on the development and survival of their offspring. Adults were maintained in synthetic seawater controls and EE2 treatments (0.005, 0.05, 0.5, 50 μg/L) over a sixteen week period. Egg capsules were collected from the adults following four, ten and sixteen weeks of adult exposure and transferred to different EE2 exposure scenarios. Treatment concentrations were selected to represent changes in EE2 exposure that could occur over different periods in an organism's lifecycle. Egg capsules laid by adults were therefore transferred to control or EE2 treatments (0.005, 0.05, 0.5, 5, 50, 500 μg/L) to develop until hatching. The percentage of egg capsules with unviable eggs and abnormalities, number of days for hatching to occur and hatching success were measured. The veliger larvae that hatched from egg capsules following two, eight and fourteen weeks of adult exposure to EE2 and controls were used in 96 h acute toxicity tests with controls and EE2 treatments at concentrations of 0.5, 5, 50, 500, 1250, 2500, 4000 μg/L. Exposure of adult *N. burchardi* and *N. jonasii* to EE2 affected the percentage of egg capsules with unviable eggs, the development and hatching success of embryos and survival of veligers. These toxicity tests produced a complex set of results with different responses in developing eggs and veliger larvae to the adult EE2 treatments and length of adult exposure. This study demonstrates the importance of trans-generational testing and adult exposure scenarios in toxicity investigations.

1. Introduction

The impact of chemical compounds which disrupt the normal functioning of hormone systems in invertebrates has been documented extensively in the induction of imposex and intersex (Bryan et al., 1986; Matthiessen and Gibbs, 1998) in over 150 species of molluscs worldwide (Matthiessen et al., 1999; Oetken et al., 2004). Population declines combined with reproductive and morphological abnormalities in gastropods and bivalves led to the identification of organotins, in particular tributyltin (TBT) as Endocrine Disrupting Compounds (EDCs) with a cause-and-effect association corroborated through field and laboratory studies (Bryan et al., 1986, 1987; Gibbs et al., 1987; Matthiessen and Gibbs, 1998). While these cases have demonstrated the susceptibility of molluscs to endocrine disruption (Matthiessen, 2008), comparatively fewer studies have been done on the effect of other known and potential EDCs, such as estrogens and estrogen mimics, on molluscs.

The best way to detect the effects of known and potential EDCs in invertebrates is via full-lifecycle or multi-generational toxicity testing (Brennan et al., 2006; Campiche et al., 2007; Dietrich et al., 2010; Ingersoll et al., 1999; Tate et al., 1997). Lifecycle and multi-generational testing allows various, potentially sensitive, periods in the life cycle such as embryogenesis, gonadal development, metamorphosis and reproduction to be studied with measurable and ecologically relevant endpoints such as growth rate, molting, behavior, reproductive output, viability of offspring and sex ratios (Brennan et al., 2006; Depledge and Billinghurst, 1999; Dietrich et al., 2010; Dodson et al., 1999; Ingersoll et al., 1999; Segner et al., 2003). Full-lifecycle tests may detect effects that are not immediately obvious and only manifest long after exposure. Trans-generational or multi-generational life cycle tests may, additionally, detect carry-over effects (Brennan et al., 2006; Cary et al., 2004), maternal transfer effects (Colborn et al., 1994; Ingersoll et al., 1999; Oberdörster et al., 2000), or sex-specific responses (Cary et al., 2004; Oberdörster et al., 2000) and make possible the calculation of population growth rates, a parameter than can be used to estimate declines or increases in a population (Forbes and Calow, 2002). To date, full-lifecycle and multi-generational toxicity tests with EDCs have been primarily focused on organisms with

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short generation times which are easy to rear in the laboratory including insects (Campiche et al., 2007; Taenzler et al., 2007; Watts et al., 2001), crustaceans (Brennan et al., 2006; Dietrich et al., 2010; Jaser et al., 2003; McKenney and Celestial, 1996; Vandenbergh et al., 2003) and nematodes (Tominaga et al., 2003).

Determining the effects of EDCs over the full life-cycle of many molluscs, including caenogastropods (older taxon name: prosobranch gastropods) that are comparatively much longer lived, is more complicated, requiring more time, effort and expense (Ingersoll et al., 1999). Nevertheless, a few authors have now published reports on the effects of EDCs on adult gastropods and their developing embryos and juvenile offspring up to the time of sexual maturity and reproduction (Czech et al., 2001; Leung et al., 2004; Oehlmann et al., 2000; Tillmann et al., 2001). In cases where full life-cycle tests are not possible for some mollusc species, shorter exposure utilizing early life stages (eggs, embryos and larvae) are a more feasible option. These early life history stages are often found to be more sensitive to toxicants, pollutants and other environmental parameters (Ringwood, 1990) and therefore are likely to be susceptible to the effects of EDCs.

The effects of known and potential EDCs on larval and juvenile life history stages of molluscs have been investigated using oysters (Andrew et al., 2008; Inoue et al., 2004; Lawler and Aldrich, 1987; Nice et al., 2000), mussels (Beaumont and Budd, 1984), clams (Coelho et al., 2001; Laughlin et al., 1989; Ruiz et al., 1995), and economically important gastropods such as abalone (Conroy et al., 1996; Shofer and Tjeerdema, 2002). Many of these tests have focused on organotin compounds (Beaumont and Budd, 1984; Duft et al., 2003; Inoue et al., 2004; Laughlin et al., 1989; Lawler and Aldrich, 1987). Less work has been done on the sensitive early life stages of prosobranch gastropods; i.e. caenogastropods (e.g. Sousa et al., 2005) even though in many cases the adults have been found to be affected by EDCs (Benstead et al., 2011; Bryan et al., 1988; Duft et al., 2007; Jobling et al., 2004; Leung et al., 2004; Oehlmann et al., 2000, 2006; Schulte-Oehlmann et al., 2000; Tillmann et al., 2001).

The exposure of some vertebrate and invertebrate organisms to EDCs in adulthood has been shown to manifest in different ways in the early life stages of their offspring, often with permanent and irreversible consequences (e.g. Colborn et al., 1994). Additionally, some EDCs and toxic compounds have been found to produce widely differing responses in the offspring or subsequent generations of the exposed organisms (Campiche et al., 2007), including increased (Brennan et al., 2006) or decreased (Oehlmann et al., 2000) sensitivities to the compounds. Exposure of adults may also result in maternal transfer effects, where female organisms expel or transfer contaminants out of their body tissues into their eggs or offspring (e.g. McClellan-Green et al., 2007; Oberdörster et al., 2000). Relying only on data from adult exposure or from one discreet stage in the life cycle of an organism may under or overestimate potential effects later in the life of the organism or its progeny (Brennan et al., 2006; Campiche et al., 2007; Dietrich et al., 2010; Tate et al., 1997). There is thus a pressing need to focus on determining the effects of EDCs on sensitive stages of caenogastropods including embryos and developing larvae as well as determining the potential for trans-generational effects following adult exposure.

The synthetic estrogen 17α-ethynylestradiol (EE2) is the active component of the birth control pill and an EDC with estrogenic properties (Jaser et al., 2003). EE2 has been detected in the influents and effluents of sewage treatment plants worldwide, as well as in rivers and coastal and estuarine waterways (Ying et al., 2002). EE2 has been recorded in effluent at concentrations up to 42 ng/L and in surface waters at concentrations up to 5.1 ng/L (Ying et al., 2002). Most typically, however, EE2 concentrations in effluent are below 2 ng/L and surface waters in the range of 0.1–0.5 ng/L. Braga et al. (2005) detected EE2 at concentrations as high as 0.5 ng/g in sediment nearby to a deep ocean sewage outfall in Australia. The existence of EE2 in coastal and marine environments, therefore, has the potential to impact upon organisms such as aquatic gastropods.

The aim of this study was to determine the impacts of the exposure of EE2 on two species of *Nassarius* snails and on the early life stages of the progeny and to determine whether a carry-over effect in offspring occurs following the long term exposure of adults to EE2. The test organisms, *Nassarius burchardi* and *Nassarius jonasii*, are estuarine sediment dwelling gastropods endemic to waters around eastern and southern Australia. It was predicted that if adult *Nassarius* are exposed to EE2 there will be carry-over effects in their embryos and larvae and the longer the adults are exposed the more pronounced the effects in their offspring will be.

2. Materials and methods

2.1. Collection and acclimation of test organisms

Adult *N. burchardi* and *N. jonasii* were collected from the Jervis Bay Marine Park, NSW, Australia (35°03′S, 150°44′E). Snails were returned to the laboratory and placed in aquaria after sorting to species and measured to ensure there were no differences in the size frequency distribution of the specimens selected for toxicity testing. The snails were introduced to a feeding regime of frozen fish meat (Fish Fuel Co.™ Marine Food) and aquarium algal wafers (Hikari®), and gradually acclimated, over a four week period, to Crystal Sea® Marinemix synthetic seawater (Specific Gravity, S.G. 1.022), a temperature of 20 ± 1 °C and a 12-h light:dark cycle. Synthetic seawater was selected to eliminate the possibility of pollutants and to allow for greater consistency of water quality parameters. The synthetic seawater was made up following the directions provided by the manufacturer.

2.2. Preparation and validation of test solutions

17α-ethynylestradiol (98 percent, CAS: 57-63-6) was purchased from Sigma Aldrich. Stock solutions for tank dosing were prepared by dissolving nominal quantities (2.5 mg and 4.0 mg) of EE2 into one litre acid washed glass volumetric flasks with synthetic seawater. The solutions were prepared 3–5 days in advance of dosing to allow time for complete dissolution of EE2 and negate the need to use a solvent. Dissolution of EE2 was facilitated by sonication. Flasks containing stock solutions were covered in foil and stored in a refrigerator prior to use. Stock solutions were analyzed prior to use by High Performance Liquid Chromatography. Chromatographic separations were performed on a Shimadzu LC system, incorporating a LC-10AT*VP* pumping system, SIL-10AD*VP* auto injector, DGU-14A online degasser, SPD-M10A*VP* diode array detector (201 nm) and Shimadzu Class-VP software on a Pentium III 700 MHz processor. Quantification of EE2 was performed using an Aqua C18 125A column (5 μm particle diameter, 150 mm × 4.6 mm, Phenomenex) on a methanol:water gradient separation at a flow rate of 1 mL/min (Table 1, Supplementary material). For 2.50 mg/L nominal concentration of stock solution the average quantified concentration was 2.53 mg/L ± 0.03 ($n=9$). For 4.00 mg/L nominal concentration of stock solution the average quantified concentration was 4.05 mg/L ± 0.01 ($n=3$). Previous analyses determined EE2 did not accumulate in the sediment of the tanks but was detected in the aqueous phase. Stock solutions were diluted in volumetric flasks of synthetic seawater to achieve the concentrations required for each experimental treatment.

2.3. Exposure of adult N. burchardi and N. jonasii to 17α-ethynylestradiol

Adults were divided into groups of 20 individuals and placed in borosilicate glass tanks with 300 g (wet weight) of washed, sieved sediment (< 450 μm grain size) and 1 l of synthetic seawater. The snails were allowed a further three weeks to acclimate in the test tanks before dosing commenced. The water in each of the tanks was dosed by undertaking a complete water change to achieve the treatment condition i.e. synthetic seawater (controls) or synthetic seawater dosed with EE2 at concentrations of 0.005, 0.05, 0.5 and 50 μg/L for a sixteen week period. All treatments had been prepared in volumetric glassware to ensure the correct concentration was dosed in the tanks. Tanks were aerated gently and re-dosed with EE2 weekly with complete water changes. During water changes the overlying water was slowly decanted from the tanks into individual containers in order to limit disturbance of sediment. The snails were fed with the equivalent of 240 mg of fish meat twice per week and 30 mg of algae wafers once per week. This feeding regime ensured that there was no leftover food to lead to spoiling in the tanks. There were three replicate tanks for each treatment and control (15 tanks in total, $n=3\times5$), for each of the two species. The egg capsules produced by the adults were collected for trans-generational egg capsule toxicity tests after four, ten and