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Multi-generational effects of four selected environmental oestrogens on *Daphnia magna*

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Abstract

The objective of this study was to determine whether vertebrate-type oestrogens have ecotoxicological effects on a crustacean species. The effects of 17β-oestradiol (E2), diethylstilbestrol (DES), bisphenol A (BPA) and 4-nonylphenol (4-NP) on the freshwater invertebrate *Daphnia magna* were assessed over first and second generations. The acute EC_{50} 48 h, based on immobilisation, for E2, DES, BPA and 4-NP were 2.87 mg/l, 1.55 mg/l, 7.75 mg/l and 0.13 mg/l, respectively. The impact of the test chemicals on moulting frequency was also assessed. The EC_{50} 48 h, based on the inhibition of moult number for E2, DES and 4-NP were 2.04 mg/l, 1.87 mg/l and 0.14 mg/l, respectively. BPA was not observed to impact the moulting frequency of *D. magna* at concentrations tested. In a series of separate studies, the effects of the four selected test compounds on the survival, moulting frequency and reproduction of first and second generational *D. magna* were assessed over a period of 21 d. Exposure of *D. magna* to 4-NP decreased the number of offspring produced in both first and second generation testing. DES proved to have no significant ($p \leqslant 0.05$) inhibition of fecundity in first generation but when second generation daphnids were exposed to DES, a significant ($p \leqslant 0.05$) reduction in the number of offspring was recorded. When *D. magna* were exposed to E2 or BPA, no statistically significant ($p \leqslant 0.05$) inhibition in the number of moults or offspring produced was observed.

Keywords: *Daphnia magna*; Oestrogens; Moulting; Reproduction

1. Introduction

In recent years there has been growing concern about the release of certain chemicals into the environment which may be altering the reproductive health of humans and their endocrine systems (Sharpe and Skakkebaek, 1993; Sharpe, 2001). Environmental oestrogens are man-made and naturally occurring chemicals, which in the environment can mimic or interfere with the binding and action of natural hormones, thus disrupting physiological processes. Various structurally diverse chemicals have been shown to be capable of binding to the oestrogen receptor and eliciting an oestrogenic response. The list of known oestrogens is extensive and includes natural and synthetic hormones, pesticides, pharmaceuticals and chemicals used in the manufacture of paints and plastics. The issue of endocrine disruption is not a new one with evidence of the disruption of the endocrine systems of wildlife being well documented. Molluscs (Minchin et al., 1995), alligators (Guillette et al., 1994) and fish (Purdom et al., 1994; Sumpter, 1995) are just a few examples of wildlife which have been adversely affected. Despite the fact that invertebrates constitute circa 95% of all animal species and are key components of all ecosystems (Pinder et al., 1999), studies of the effects of endocrine disrupting chemicals have focused mainly on the in vivo assessments in mammals, reptiles and fish.

In crustaceans many physiological processes are regulated by neurohormones including lipid metabolism, ionic balance, moulting, growth, regeneration, gonadal development, reproductive physiology, digestion and cardiac activity (DeFur et al., 1999). The majority of invertebrate

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hormones identified are peptide neurohormones. There is a paucity of evidence for the direct action of endocrine disrupting chemicals in peptide-based signalling systems (Pinder et al., 1999). However, key insect and crustacean hormones are non-peptide based (e.g., ecdysteriods, juvenile hormones) and processes in which these hormones are involved may conceivably be more susceptible to receptor related interference. Although vertebrate-type steroids have been detected in crustacean tissues, their functional significance has yet to be confirmed. The likely impact of interference at any particular locus within the endocrine system of invertebrates is difficult to predict. It is probable that the disruptive effects encompass reproduction, moulting, feeding and behaviour (Pinder et al., 1999). Baldwin et al. (1995) examined the effects of diethylstilbestrol (DES) on *D. magna* to try to identify the physiological target sites of xenoestrogens. They found that *D. magna* exposed to mg/l concentrations of DES resulted in a decreased moulting frequency among first generation juvenile and decrease fecundity in second generation daphnids. 4-nonylphenol (4-NP) was previously reported to reduce fecundity in first generation *D. magna* at 50 and 100 μg/l (Baldwin et al., 1997). Second generation studies (at a top concentration of 6.2 μg/l) were shown to elicit no adverse effects on survival and offspring production (Baldwin et al., 1997).

Daphnia magna are freshwater invertebrates of the order Cladocera, more commonly know as the water flea. They have a relatively short life (7–8 weeks at 20 °C) and reach sexual maturity within 6–8 d of leaving the brood chamber (Ten Berge, 1978). In culture, reproduction is limited to the production of females by diploid parthenogenesis. Males are only produced in response to an environmental stress, such as high population densities, accumulation of excretory products and/or a decrease in available food. Due to their size, ease of culture and short life span, *D. magna* are extensively used as a representative freshwater invertebrate species in toxicological studies. The body of a *Daphnia* is not clearly segmented, with the thoracic and abdominal regions covered in a secreted shell or carapace. Although this carapace has a bivalve appearance, it is in fact a single fold which drapes ventrally over the organism (Pennak, 1989). In *D. magna*, as in all crustaceans, growth occurs within a few minutes of discarding the carapace before the exoskeleton hardens and loses its elasticity, thus moulting is an important physiological process in *Daphnia* (Pennak, 1989). It has been hypothesised that some xenobiotics which disrupt endocrine processes in vertebrates can also interfere with the hormonally related moulting process in arthropods by acting as antagonists of endogenous ecdysteroids resulting in the blocking the ecdysteriod receptor (Zou and Fingerman, 1997).

While toxicological data exists for acute and to a lesser extent first generation chronic toxicity for oestrogen mimics, there is a paucity of published information available on multi-generational testing. The purpose of this paper is to investigate the impact of exposing *D. magna* to the four selected oestrogen mimics {17β-oestradiol (E2), DES, bisphenol A (BPA) and 4-NP} employing acute (immobilisation and moulting) and chronic endpoints (survival, moulting and reproduction). The test chemicals were chosen in an attempt to represent a selection of the structurally diverse range of oestrogen mimics typically found in the environment. E2 is the major natural reproductive hormone found in females and due to its known high oestrogenic potency, it is often selected as a positive control in oestrogenicity testing. DES is a synthetic hormone which was given to women in the 1950s to prevent miscarriage and was also used as a growth promoter in cattle. BPA is a plastic monomer used in the manufacture of polycarbonated plastics. 4-NP is an alkylphenol ethoxylate which are the second largest group of non-ionic surfactants in commercial production. In addition to the standard acute and chronic endpoints (immobilisation, mortality and reproduction) moulting frequency was also investigated. Moulting behaviour is a toxicological endpoint which has been shown to be adversely affected by endocrine disrupting chemicals (Baldwin et al., 1995; Zou and Fingerman, 1997). In this study, the use of moulting frequency as a sub-lethal indicator of an acute and/or chronic toxicity is evaluated for the aforementioned test chemicals.

2. Materials and methods

2.1. Chemicals

17β-Oestradiol, diethylstilbestrol and bisphenol A were supplied by Sigma Aldrich Ireland Ltd., Ireland. 4-Nonylphenol was supplied by Lancaster Ltd., Germany. The test chemicals were dissolved in ethanol and added to culture medium to deliver the desired concentration. The concentration of solvent in the stock solution did not exceed 0.1 ml/l in the highest exposure solution. Solvent controls were included in all testing. Analytical grade potassium dichromate (Timstar Laboratory Supplies Ltd., England) was used as the reference chemical to validate acute test procedures. Actual concentrations were inferred and not verified.

2.2. Test organisms

D. magna Straus stocks were originally obtained from the US EPA (Cincinnati, OH). Stocks of *Daphnia* were cultured in dilution water as per International Organisation for Standardisation (ISO) guidelines (ISO, 1996). Culture vessels consisted of 1 l beakers containing 40 *Daphnia*. The culture medium was renewed three times weekly. Daphnids were fed as previously described by Olmstead and LeBlanc (2000) the unicellular algae *Chlorella vulgaris* supplemented with Tetrafin fish food (Tetra GmbH, Germany). The fish food supplement was prepared by homogenising 2.5 g of fish food in 250 ml of water. Solids were