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Reproductive and developmental effects of endocrine disrupters in invertebrates: in vitro and in vivo approaches

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Abstract

In order to gain basic understanding in the ecotoxicity of endocrine disrupting chemicals or EDCs (including natural chemicals and some pharmaceuticals), many international research groups are currently testing these chemicals using aquatic invertebrates. This paper discusses relevant examples to address key questions: which aquatic invertebrates are likely to be vulnerable to mammalian and non-mammalian EDCs; and which types of invertebrate chronic tests might be most sensitive and cost-effective to address potential environmental exposures? For a full review of invertebrate endocrine disrupter research see Endocrine Disruption in Invertebrates: Endocrinology, Testing and Assessment (1999). As an example, crustaceans are a particular focus of EDC research, reflecting their abundance in nature, commercial importance and their inclusion in the regulatory assessment schemes for active pharmaceutical ingredients (APIs). There is a diverse literature on the developmental and reproductive effects of mammalian EDCs in Crustacea, although there is growing evidence that such effects are probably not mediated via arthropod hormone systems. For example, recent studies in Europe using a marine copepod (*Tisbe battagliai*) life-cycle test have evaluated ecdysteroid agonists (e.g. 20-hydroxyecdysone), oestrogen agonists (e.g. diethylstilbestrol (DES), 17β-oestradiol, oestrone and 17α-ethynylestradiol) and the pharmaceutical anti-oestrogen (ZM189, 154). While 20-hydroxyecdysone and DES were highly toxic, the other compounds tested show no significant toxicity to copepods. Furthermore, in vitro studies indicate that these environmental EDCs and several related APIs are not active against the ecdysteroid receptor. Therefore, other undefined modes of action appear to be responsible for crustacean toxicity in vivo and caution should be exercised before ascribing any apical effects to endogenous endocrine mechanisms, or before crustacean 'EDC' data are extrapolated to other invertebrate taxa. © 2002 Published by Elsevier Science Ireland Ltd.

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1. Introduction

Invertebrates comprise approximately 95% of all terrestrial and aquatic animal species (Wilson, 1999), therefore it is clearly necessary to take this

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biodiversity into account when addressing the potential developmental and reproductive hazards posed by environmental EDCs. While knowledge into potential endocrine-mediated impacts in invertebrates is growing steadily (DeFur et al., 1999), practical and scientific limitations in ecotoxicology are such that it remains necessary to continue to extrapolate from laboratory data based on a few invertebrate species to the wider range of invertebrate species in nature. In many cases, the aquatic organisms used for regulatory ecotoxicity testing purposes have been selected, at least in part, from knowledge of aquaculture (e.g. rotifers, daphnids and bivalve molluscs) and therefore may not be ideal representatives of the major invertebrate groups (Ingersoll et al., 1999).

The concern over endocrine disruption in aquatic invertebrates, additional to the international debate over EDCs and human health, has been highlighted by the example of tributyltin (TBT)-induced reproductive damage and population declines in molluscs (reviewed by Matthiessen and Gibbs, 1998; Alzieu, 2000). From the initial observations of dramatic impacts in French oyster populations as early as 1975, the biochemical and physiological mechanisms of TBT action remains the subject of on-going research. Based on evidence that exposure of molluscs to TBT is associated with an increase in testosterone levels in females and induction of imposex (penis growth in females), it is considered most likely that TBT may be inhibiting cytochrome P450 aromatase (Matthiessen and Gibbs, 1998; Alzieu, 2000; Vos et al., 2000). Other factors thought to contribute to TBT-induced imposex in gastropods include the inhibition of testosterone excretion and suppression of release of a neuroendocrine factor from the pleural ganglia (Matthiessen and Gibbs, 1998; DeFur et al., 1999). This example underscores the fact that the developmental and reproductive health of invertebrates is dependent upon a highly integrated neuroendocrine system, as is the case for all animals (Laessig et al., 1999). Interestingly, attention has been raised regarding antidepressants and their potential reproductive toxicity in molluscs due to neuroendocrine disruption (Fong, 2001).

2. Invertebrate diversity

In large part due to the TBT example outlined above, there is a significant international research effort into the impacts of mammalian EDCs on aquatic invertebrates and the underlying modes of action (MOA) in non-target organisms. Of the major evolutionary invertebrate phyla (Fig. 1, adapted from Solomon et al., 1996), those most widely used for chronic ecotoxicity testing of potential EDCs include: arthropods—namely, crustaceans and insects; annelids—including earthworms and polychaetes; and molluscs—including gastropods and bivalves (DeFur et al., 1999). In part prompted by available genomic information, there is also growing interest in using the Pseudocoelomate nematode, *Caenorhabdites elegans* in ecotoxicity testing (Anderson et al., 2001; endocrinology reviewed by DeFur et al. (1999)).

Although of the same evolutionary lineage as the mammals and other Chordates, echinoderms are not widely used in ecotoxicity testing. Nonetheless, basic research shows that the mammalian steroids are important in echinoderm reproductive endocrinology (DeFur et al., 1999;

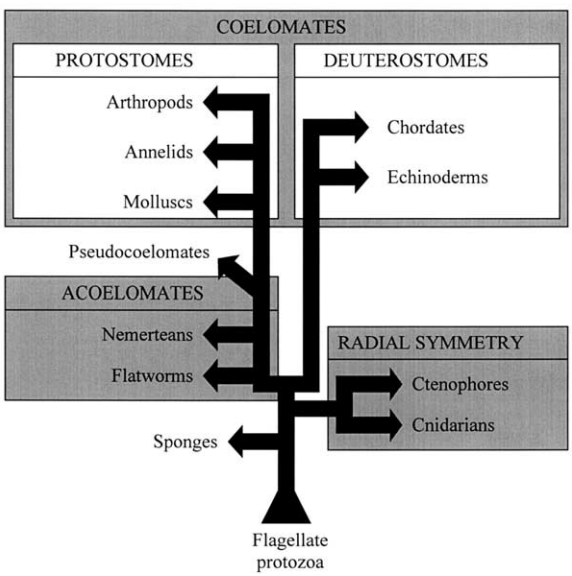

Fig. 1. Phylogenic relationships within the animal kingdom (adapted from Solomon et al., 1996).

Wasson et al., 2000) and limb development (Carnevali et al., 2001), while echinoderm embryos have been used in pharmaceutical research (Vogel et al., 1995; Pagano et al., 2001). DeFur et al. (1999) provide a comprehensive review of the basic endocrinology of all the major and minor invertebrate phyla.

Overall, there are examples of environmental chemicals affecting development and reproduction in many invertebrate groups, however, in many cases the only mechanistic information available is that inferred from mammals and fish. Progress in understanding EDCs and appropriate extrapolation of invertebrate chronic toxicity data to the wider world critically depends upon understanding MOA in a taxonomic context. This need for scientifically based extrapolation underscores the importance of the Weybridge workshop's definition of an endocrine disrupting chemical as being 'an exogenous substance that causes adverse effects in an intact organism, or its progeny, consequent to changes in endocrine function' (European Commission, 1996). Given the extensive use of arthropods (e.g. amphipods, chironomids and daphnids) in the environmental hazard assessment of active pharmaceutical ingredients (APIs) and other synthetic chemicals, the subsequent discussion uses relevant examples from the available literature to highlight the need for a MOA approach to ecotoxicity testing for potential invertebrate EDCs.

3. Ecotoxicity testing with crustaceans and insects

Both laboratory ecotoxicity data and field observations relating to abnormal sexual development in crustaceans and insects are reviewed by DeFur et al. (1999). Moreover, many environmental testing schemes for APIs or for effluents from pharmaceutical manufacturing plants use test protocols with daphnids and other aquatic organisms. The endocrinology of crustaceans and insects is reviewed by DeFur et al. (1999) with a clear emphasis on the functional importance of ecdysteroids and juvenile hormone (JH).

3.1. Amphipods

A number of standardised laboratory protocols exist for chronic toxicity testing using marine (e.g. *Corophium volutator*) and freshwater species (e.g. the amorous *Gammarus* sp., *Hyalella* sp., etc) (Ingersoll et al., 1999). Recently, Gross et al. (2001) reported abnormalities in sexual development of the amphipod *Gammarus pulex* (L.) found below sewage treatment works in England. They observed a highly significant number of abnormal females (abnormal structure of oocytes in vitellogenesis) collected from a site known to elicit high oestrogenic responses in fish. Amphipod body size was significantly shorter and male:female size differential was significantly reduced below one of the discharges. Watts et al. (2002) exposed laboratory populations of *G. pulex* to 17α-ethinylestradiol (EE2) (0.104–7.5 μg/l) for 100-day, observing significant increases in population size and in the proportion of female amphipods at the higher EE2 exposures.

3.2. Chironomids

Freshwater chironomids are widely used in Europe and North America for assessing the toxicity of contaminated sediments. Watts et al. (2002) described chronic exposure of *Chironomus riparius* to EE2 and Bisphenol A (BPA) over two generations in chronic sediment exposure assays. Results showed that emergence time and percentage adult emergence were affected by EE2 and BPA exposure. These effects were primarily associated with the second generation of test animals, most notably in the BPA study, where the emergence of male and female adults was significantly ($P < 0.05$) delayed at concentrations ranging from 78 ng/l to 0.75 mg/l. At very low concentrations (1 ng/l) of EE2, both the first and second generation of adults emerged significantly earlier than control animals. The authors noted, however, that although certain responses were significantly affected, their results in general do not suggest that the apical criteria examined could be used to detect the oestrogenic effects of EE2 and BPA on chironomids.

Also, Meregalli and Ollevier (2001) exposed *C. riparius* larvae to EE2 (1, 10 and 100 μg/l as nominal concentrations) but saw no significant impacts in survival and mouthpart deformities.

3.3. Cladocerans

For several decades, species such as *Daphnia magna* have been widely used for regulatory environmental assessments of APIs and other chemicals, including some compounds that are established as EDCs in fish and mammals. For example, laboratory studies by Baldwin et al. (1995) demonstrate reduced moulting frequency in daphnids exposed to diethylstilbestrol (DES) (500 μg/l) but no reduction in survival or fecundity. Exposure to nonylphenol and its related polyethoxylate has also been shown to affect daphnid fecundity and sex determination, while studies on many other environmental contaminants and daphnids are reviewed by Ingersoll et al. (1999).

More recently, Olmstead and LeBlanc (2001) reported methoprene (a JH agonist) toxicity to *D. magna*, observing multiple mechanisms of toxicity and low-exposure concentration effects. Peterson et al. (2001) also exposed *Daphnia pulex* to the methoprene (10 and 100 μg/l) and saw effects in terms of the incidence of all-male broods compared with controls. Since methoprene has been found to bind to the mammalian retinoid × receptor, Peterson et al. also tested the effects of retinoic acid on daphnid reproduction; however, neither 9-*cis*-retinoic acid nor all-*trans*-retinoic acid had any observable effect. Exposure of daphnids to 20-hydroxyecdysone (1, 10 or 100 μg/l) suggest that JH and ecdysteroids might play a role in daphnid sex determination.

3.4. Copepods

Prompted by reports of abnormal intersex copepods found near a UK sewage effluent discharge, Hutchinson et al. (1999a,b) used a marine copepod (*Tisbe battagliai*) 21-day life-cycle test to evaluate ecdysteroid and oestrogen agonists and a pharmaceutical anti-oestrogen (ZM189, 154). While 20-hydroxyecdysone and DES were highly toxic (21d LC50 values of 53.4 and 31.6 μg/l, respectively), compounds such as 17β-oestradiol and oestrone (10–100 μg/l) produced no adverse effects in copepod populations. Bechmann (1999) also demonstrated the toxicity of nonylphenol (NP) to *T. battagliai*, however, no mechanism-specific data were provided. More recently, Andersen et al. (2001) studied the copepod (*Acartia tonsa*) larval development during exposure to known vertebrate steroid hormone agonists (including BPA, oestradiol, oestrone, 17α-ethinylestradiol, *p*-octylphenol, progesterone and testosterone) and antagonists (including flutamide and tamoxifen). Of the natural chemicals and pharmaceuticals studied, potent inhibitors of naupliar development (based on 5d EC10 nominal values) were: EE2 (46 μg/l), octylphenol (5.2 μg/l) and tamoxifen (8.7 μg/l). Testosterone and progesterone did not inhibit development, however, based on 5d EC10 nominal values, inhibitory responses were seen for flutamide (135 μg/l) and hydroxyflutamide (5.3 μg/l). The ecdysteroid agonist 20-hydroxyecdysone did not produce any effect up to a nominal concentration of 1.5 mg/l. Finally, Brietholz and Bengtsson (2001) reported at absence of significant chronic effects in *Nitocra spinipes* exposed for 18 days to either DES (0.3–30 μg/l), EE2 (0.5–50 μg/l) or E2 (0.5–50 μg/l).

3.5. Decapods

Prompted by interest in biofouling, a number of research groups have addressed endocrine mechanisms of development in barnacles. Interest has extended to environmental oestrogens, especially the effects of 4-*n*-nonylphenol (NP) and 17β-oestradiol (E2) on vitellin-like protein induction and settlement of *Balanus amphitrite* and early development of *Elminius modestus* (Billingshurst et al., 1998, 2000). Barnacle early life-stages (nauplii and cyprids) were exposed in the laboratory to NP or E2, both compounds causing disruption of the timing of larval development with evidence that the timing of exposure is critical and exposures for a period of 12 months caused long-term effects. Based on these and related studies, Billingshurst et al. suggest that vitellin-like proteins may act as biomarkers for oestrogen exposure in oviparous crustaceans.

3.6. Mysids

In view of their importance in marine food chains, test protocols have been successfully developed in North America for *Americamysis bahia* (previously *Mysidopsis bahia*). Mysids have been shown to be especially sensitive to pesticides such as methoprene (reviewed by Ingersoll et al., 1999). There appear to be no data available from mysid tests on APIs or related compounds, perhaps due to the practical limitations of conducting high volume flow-through tests with expensive pharmaceutical compounds.

4. In vitro studies and mechanistic data

While the crustacean and insect database for the apical developmental and reproductive effects of mammalian EDCs is greater than for other invertebrate taxa, there is no known functional role for mammalian-type steroids in arthropods (DeFur et al., 1999). This analysis has led to recent multi-disciplinary links between insect biochemists and ecotoxicologists, with the aim of using in vitro methods to identify true arthropod EDCs. Recently, Dinan et al. (2001) have used an in vitro ecdysteroid receptor-based (EcR) screening assay to evaluate a wide range of compounds, including mammalian steroids and some related APIs. All of the oestrogens tested (including DES and alkylphenols) showed no response in vitro, suggesting that the toxicity of these compounds to crustaceans (see above) is not mediated via the EcR. Compared with the APIs tested in crustaceans in vivo, Dinan et al. observed very weak antagonistic activity for EE2 only (equivalent to mg/l levels). In terms of oestrogen antagonists, tamoxifen has not been tested using the EcR screen, however, Pagano et al. (2001) suggest that the embryo toxic effects seen in marine echinoderms may be mediated via oxygen radical over production that occurs in tamoxifen metabolic activation. Whether this mechanism is relevant to copepods (Andersen et al., 2001) or other invertebrate taxa remains to be studied. Further work is needed using a combination of such in vitro and in vivo methods, ideally including use of the reference EDCs recommended by Ingersoll et al. (1999) (Table 1).

Table 1
Suggested reference chemicals for evaluating relative endpoint or invertebrate species sensitivity to potential EDCs (after Ingersoll et al., 1999)

Possible MOA	Chemical
JH agonist	Methoprene
JH antagonist	Precocene
Ecdysone agonist	20-Hydroxyecdysone
Ecdysone antagonist	Homobrassinolide, luteolin
Aromatase inhibitor	Fadrozole
Androgen agonist	Methyltestosterone
Androgen antagonist	Flutamide
Oestrogen agonist (weak)	4-*Tert*-pentylphenol
Oestrogen antagonist (strong)	17α-Ethinylestradiol
Oestrogen antagonist	ZM189, 154

5. Conclusions and recommendations

As in other aspects of ecotoxicology, attempts to help protect natural invertebrate populations from the potential adverse effects of synthetic chemicals involves testing using a limited suite of species followed by extrapolation to the wider world via the predicted no-effect concentration (PNEC). For potential EDCs, including a minority of APIs, such extrapolation will be most effective if there are robust chronic test protocols available using a variety of species that encompass the reproductive endocrine systems present in the invertebrate fauna (Hutchinson et al., 2000). At present, there is a limited but nonetheless useful range of test methods available for measuring the developmental and reproductive effects of APIs and related chemicals to a given invertebrate group (e.g. copepods, daphnids or oyster embryos) (DeFur et al., 1999). However, as exemplified by the historic failure of acute toxicity tests to predict the chronic toxicity of TBT to marine molluscs, the greater challenge lies in having a sufficiently robust understanding of the MOA that will ensure accurate prediction of the PNEC for the relevant ecosystem. While this is a significant task, it is argued that the question of EDCs

and invertebrate health has arguably helped to establish a new chapter of 'mechanistic ecotoxicology' to better avoid potential TBT scenarios of the future. In the long-term, a MOA approach to species selection for API testing is likely to be more cost-effective and may also have ethical benefits in reducing the need for vertebrate tests (Vogel et al., 1995). More specifically, for the environmental assessment of potential invertebrate EDCs, including neuroendocrine disrupters, it is recommended that attention be given to: (1) using reference compounds to validate new invertebrate tests methods and effect endpoints (Table 1); (2) evaluating in vitro data from both mammalian-type and arthropod hormone receptor binding assays; (3) utilising small-scale test methods which do not generate effluent handling problems or use large quantities of APIs; (4) using environmentally relevant test concentrations and exposure routes, including supporting chemical analyses; (5) considering improved test methods using species for which molecular tools are becoming available (e.g. the nematode *C. elegans*). Finally, for the future environmental assessment of APIs, whatever their MOA, it is also highly desirable that emerging regulatory schemes are cost-effective and actively encourage an ethical approach to ecotoxicity testing. To achieve these aims, stakeholders in the environmental safety assessment of human and veterinary APIs need to allow the future use of validated novel ecotoxicity (especially invertebrate) testing methods that will improve upon today's limited range of standard protocols.

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