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Endocrine disruption horizon scanning: aquatic invertebrates review

Science Report – SC030276/SR1

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Steve Killeen

Head of Science

This document is one of four reports produced under the *Endocrine disruption horizon scanning* project (SC030276), which is part of Environment Agency's R&D Project Initiation Document P6-020/U, *Development of methods for detection of endocrine disruption and application to environmental samples*.

The aim of the *Horizon scanning* project is to identify and review new and emerging aspects of endocrine disruption (ED). The full list of documents in the project is:

- *Endocrine disruption horizon scanning : Aquatic invertebrates review (SC030276/SR1)*
- *Endocrine disruption horizon scanning: Molecular and genomic contributions (SC030276/SR2)*
- *Endocrine disruption horizon scanning : Priority and new endocrine disrupting chemicals (SC030276/SR3)*
- *Endocrine disruption horizon scanning: Current status of endocrine disruptor research and policy (SC030276/SR4)*

Executive summary

Previously, the Environment Agency's risk assessment of endocrine disrupting chemicals (EDCs) in rivers has concentrated on the risks posed to fish, as part of its remit to protect fish populations. A large number of studies have shown that fish are affected by EDCs in rivers.

However, there are a several reasons to reduce the effort being spent on fish and move instead to studying effects on invertebrates.

Firstly, EDC impacts on the invertebrate fauna of UK rivers and lakes is generally less well documented. This raises the question of whether a risk strategy based only on fish is protective of the wider environment.

Secondly, one of the main gaps in endocrine disruption (ED) research is the link between individuals and populations, and there are difficulties associated with addressing population level effects in fish that are less problematic with invertebrates. For example, invertebrate life cycles are much shorter and therefore easier to monitor over several generations; in addition, invertebrates tend to be less mobile than fish and may reveal localised impacts.

Finally, there is general call to reduce the amount of experimental work (or testing) that uses vertebrates. If invertebrate species can be used instead of fish, without diminishing the information gained, they should be used for ethical reasons.

To this end, this report reviews the most recent advances in research on ED in invertebrates.

In 1999, the Environment Agency commissioned a review of the literature on ED in invertebrates. This report provides an update on the 1999 review, examining the literature from 1999 to 2007. It also explores whether any of the knowledge gaps identified or recommendations made in the 1999 report have been addressed in the last seven years.

The main findings of this review are as follows:

- Research into ED in invertebrates has continued at a steady pace since 1999.
- A considerable proportion of that effort has been spent on simple concentration and effect studies, either using new chemicals on well-established test organisms (such as *Daphnia* or mysids), or using well-tested EDCs (such as tributyl tin (TBT) or nonylphenol) on new species. The value of this research is questionable.
- Although there have been some advances since 1999, our understanding of invertebrate endocrine systems remains generally poor. This gap was highlighted in the 1999 report and remains to be filled. A large proportion of studies state ED as a reason for the effects recorded, but this is generally an assumption rather than a proven fact. Furthermore, although the effects reported have been attributed to ED, these could potentially be due to a number of toxicological responses.
- Some advances have been made in our understanding of the mollusc endocrine system, and this may provide a way forward for the use of molluscs as environmental monitors or test organisms for ED.
- The majority of ED research on invertebrates has been laboratory based. There are comparatively few studies of ED in invertebrates living in their natural environment.
- Little is known about the potential 'real-world' effects of ED. With the exception of the well-known impacts of TBT on gastropods, there is little evidence demonstrating

ED impacts on natural invertebrate populations. This is another gap that was identified in 1999, which has not been addressed in the last eight years.

- Some progress has been made towards the use of new testing and monitoring species (such as gastropods). But in general, research remains focussed on normal laboratory testing species (that is, *Drosophila sp.*, *Daphnia sp.*, mysids and copepods). Some progress has been made using other phyla (such as the Echinodermata, Nematoda and Annelida), but this has tended to focus on concentration and effect rather than mechanisms.
- Daphnids, copepods and mysids remain important laboratory species for EDC testing and screening strategies, such as those of the United States Environmental Protection Agency (US EPA), and the Organisation of Economic Cooperation and Development (OECD).

To summarise, all of the gaps in ED research identified in 1999 remain today to some extent. Our knowledge remains biased towards dose-response relationships, species sensitivities and laboratory-based exposure studies.

Thus, we still need to gain a better understanding of (a) invertebrate endocrine systems in general, and (b) population level effects in the natural environment. These in turn will strengthen any subsequent ED risk assessment or strategy, as they will demonstrate (a) whether effects are occurring specifically due to ED (and not the general toxicity of the compound), and (b) whether natural populations are being affected by ED.

The more philosophical question remains of whether it is sufficient to base a risk strategy on demonstrating effects without necessarily identifying the cause, or whether it is more important to identify with certainty those chemicals acting on the endocrine system. This question is likely to remain unanswered, with progress being made to further both sides of the argument.

Contents

1 Introduction

Previously, the Environment Agency's assessment of risk posed by endocrine disrupting chemicals (EDCs) in rivers has concentrated on fish. Fish are clearly affected by exposure to EDCs; however, the question remains as to whether a risk assessment based entirely on fish is protective of the wider aquatic environment. There is a large literature demonstrating that aquatic invertebrates are also affected by EDCs, and ongoing Environment Agency research is examining whether certain gastropod species could be useful tools to monitor biological effects of EDC exposure.

This comprehensive review brings together recent evidence of ED exposure in aquatic invertebrates, and will allow the Environment Agency to assess whether its current endocrine disruption (ED) strategy is sufficiently protective and sensitive.

Background

In 1999, the Environment Agency commissioned a review on current knowledge, evidence and research of endocrine disruption (ED) in aquatic invertebrates (Environment Agency, 1999; see also Depledge and Billinghurst, 1999, and Pinder and Pottinger, 1999). The review reported:

- what we know about the endocrine system of aquatic invertebrates;
- the potential of biomarkers to demonstrate ED in aquatic invertebrates;
- evidence of ED in wild populations;
- gaps in our knowledge and understanding of ED in aquatic invertebrates;
- a suggested way forward for future research and risk assessment of EDCs in the aquatic environment (Environment Agency, 1999).

As part of a larger project reviewing the risk assessment of EDCs, this report is an update and extension of the 1999 review. The report documents:

- advances that have been made in ED research in invertebrates since 1999;
- the latest evidence of ED occurring in natural populations;
- whether research needs identified in 1999 have been met;
- whether current activity in invertebrate ED research is commensurate to the scale of the issue, particularly as new European legislation (such as the Habitats and Water Framework Directives) come into force;
- any new research needs.

1.1 Review of findings from Environment Agency (1999) report

For ease of reference, the Executive Summary from the 1999 review is presented in Appendix 1.

1.1.1 Knowledge gaps identified by Environment Agency (1999)

The main knowledge gap identified by the Environment Agency (1999) review was that, despite the general concern about EDCs, extremely few cases of ED had been reported in wild invertebrate populations. Furthermore, even fewer cases of population-level effects of EDCs appeared to have been recorded. It was suggested that the lack of reported cases was due to a lack of investigations, although preliminary reports of ED (such as appendage abnormalities, induction of vitellogenesis, behavioural changes and disruption of larval settlement) were starting to be published (Environment Agency, 1999). Thus, a general lack of knowledge of EDC effects on wild populations was identified. Furthermore, the endocrine systems of the various invertebrate taxa can be quite markedly different, and thus a detailed understanding of all invertebrate endocrine systems is also lacking.

1.1.2 Recommendations from Environment Agency (1999)

Basic endocrinology

- All major invertebrate taxa should be considered to be at risk from EDCs.
- Understanding of the detailed endocrinology of an invertebrate is not required to identify that ED (for example impacted growth, reproduction, behaviour) is occurring in that organism.
- To understand the *mechanism* by which an EDC is impacting on an organism, further details on the organism's endocrinology is likely to be required.

Monitoring

- Systematic biological monitoring should be made near to discharges (riverine and marine) from sewage treatment works (STWs) to identify individual and population impacts.
- To achieve this, so-called 'sentinel' organisms need to be identified as indicators of ED. Such sentinel organisms need to show a characteristic endpoint that is indicative of ED.
- A full suite of sentinel organisms, including representatives from major invertebrate taxa (such as Crustacea, Mollusca, Insecta, Annelida, Coelenerata, Echinodermata), will be needed to encompass all likely targets.

Ecotoxicology

- Many invertebrate toxicity testing protocols will demonstrate effects from an EDC, without necessarily showing the mechanism by which that EDC is eliciting an effect.
- The question remains whether such protocols provide sufficient information for risk assessment purposes, a question potentially more policy based than scientific. For example, is it important to identify the mechanism by

which an EDC elicits its effects, or is simply the fact that it causes a detrimental effect sufficient to require a management strategy?

- Given that existing protocols require minimum further investment of time and money, while the costs to develop new protocols that demonstrate the mechanisms of ED are large, a two-pronged approach is recommended to encompass both methods.

The recommendations from the Environment Agency (1999) review are, therefore, related to two main issues: understanding of invertebrate endocrinology and associated mechanisms of ED; and identification of a suite of biomarkers for more complete biological monitoring in high risk areas.

1.1.3 Have the knowledge gaps and recommendations been fulfilled?

A review of recent advances in invertebrate endocrinology is presented at the start of each section for the representative groups. Clearly, we do now have a better knowledge of the endocrinology of certain invertebrate taxa, and this will lend itself to an understanding of the mechanisms by which EDCs elicit their effects.

However, the bigger question of whether an understanding of the actual mechanism is required to successfully manage a risk assessment of EDCs remains unanswered.

While certain organisms have received much more interest in the research of ED than others, a definitive 'suite' of sentinel organisms or biomarkers has not yet been proposed.

1.2 Other reviews

Recognising the increasing concern over EDCs, and the potential use of invertebrates in ecotoxicological testing, several reviews on aspects of ED in invertebrates have been published in the last few years (see Oehlmann and Schulte-Oehlmann, 2003; Barata *et al.*, 2004; Defur, 2004; Oetken *et al.*, 2004; Verslycke *et al.,* 2004). More recently, the journal Ecotoxicology (Volume 16, 2007) published 17 papers reviewing ED in aquatic invertebrates. Due to time restrictions, given that this review was in the final stages of editing when Ecotoxicology Volume 16 was published, it has not been possible to include all of the information presented from Ecotoxicology (2007) in this report. The main points are presented here, but for further information readers are directed to Ecotoxicology (2007).

All invertebrates have a hormone system based on internal signalling, although the complexity of that system varies greatly across the invertebrate phyla. The variety of hormones used by invertebrates includes steroids, peptides, amides and terpenes (Defur, 2004) (Table 1.1). Most research has concentrated on the moulting hormones and juvenile hormones of insects, the moulting hormones of crustaceans, and the neurohormones of molluscs (Defur, 2004; Soin and Smagghe, 2007). These hormones (and their systems) have been targeted for ED research for a number of reasons; for example, pesticides specifically target the moulting and juvenile hormones of insects, hence the requirement for detailed understanding of mode of action, sensitivity and so on. Likewise, gastropod molluscs have received much attention due to the effects of tributyl tin (TBT)-based antifouling paints on adult females. In contrast, echinoderms (and some molluscs) have been investigated because their evolutionary history is closer to that of vertebrates (and the potential similarities of endocrine systems). For example, the identification of structures thought to act as an oestrogen receptor in the

sea-hare (*Aplysia*) indicates that oestrogens and steroid receptors are quite old in evolutionary terms (Defur, 2004). However, beyond these few subject areas where research has been focussed, there is a recognised paucity of information on the effects of EDCs on invertebrates. This lack of information on EDC effects is mostly due to a dearth of information on invertebrate endocrine systems (Oehlmann and Schulte-Oehlmann, 2003; Defur, 2004; Oetken *et al.*, 2004; Verslycke *et al.,* 2004).

The lack of information on invertebrate endocrine systems was highlighted by LaFont (2000a), who reviewed and collated published information on these systems. Other publications that include (more limited) reviews of invertebrate endocrine systems include Oberdorster and Cheek (2001), Oehlmann and Schulte-Oehlmann (2003) and Verslycke *et al.* (2004). However, being reviews, these reports are merely collations of pre-published information and are not new reports. Nevertheless, they are good sources of recent information on invertebrate endocrine systems.

Table 1.1: Examples of reported hormones in different invertebrate taxa (from Defur, 2004)

Taxon	Reported hormones (example, *controlled process*)
Coelenterata	Neuropeptides (glycine-leucine tryptophan amides = GLWamides, *metamorphosis*); thyroids (thyroxine, *strobilation*); retinoids (9-cis-retinoic acid, *strobilation*)
Nematoda	Ecdysteroids (reported but *functional role questionable*); terpenoids (juvenile hormone (JH) like hormones, *growth*); neuropeptides (FMRFamide, *function unknown*)
Mollusca	Ecdysteroids (reported but *functional role questionable*); steroids (17ß-oestradiol, testosterone, progesterone, *sexual differentiation, reproduction in prosobranchs*); terpenoids (JH reported but *functional role questionable*); neuropeptides (APGWamide, dorsal body hormone (DBH), *sexual differentiation, gonad maturation, spawning*; egg-laying hormone (ELH), *spawning*; FMRFamide, *neuromodulation*; molluscan insulin-like peptides (MIPs), *growth, development, energy metabolism*)
Annelida	Ecdysteroids (ecdysone, *functional role unknown*); neuropeptides (FMRFamide, *neuromodulation*)
Crustacea	Ecdysteroids (ecdysone, *moulting, vitellogenesis*); steroids (17ß-oestradiol, testosterone, progesterone, *functional role under debate*); terpenoids (methyl farnesoate (MF), *metamorphosis, reproduction*); neuropeptides (androgenic hormone, *sexual differentiation*, *vitellogenesis inhibition*; crustacean hyperglycemic hormone family (CHH), *energy metabolism*; moult-inhibiting hormone (MIH), *ecdysteroid production*; vitellogenesis-inhibiting hormone (VIH), *vitellogenesis*)
Insecta	Ecdysteroids (ecdysone, *moulting, egg maturation*); terpenoids (JH, *metamorphosis, reproduction*); neuropeptides (adipokinetic hormone (AKH), *energy metabolism*; allatostatin and allatotropin, *JH production*; bombyxin, *ecdysteroid production, energy metabolism;* bursicon, *cuticle tanning*; diapause hormone, *embryonic diapause*; diuretic hormone (DH), *water homeostasis*; ecdysis-triggering hormone (ETH) and eclosion hormone (EH), *ecdysis behavior*; FMRFamides, *neuromodulation*; prothoraciotrophic hormone (PTTH), *ecdysteroid production*)
Echinodermata	Steroids (progesterone, testosterone, 17ß-oestradiol, oestrone, *vitellogenesis, oogenesis, spermatogenesis, spawning*); neuropeptides (gonad-stimulating substance (GSS), *spawning*; maturation-promoting factor (MPF), *fertilisation*)
Tunicata	Steroids (testosterone, 17ß-oestradiol, *oogenesis, spermatogenesis, spawning*); neuropeptides (gonadotropin-releasing hormone analogue, *gonad development*); thyroids (thyroxine, *probably tanning process during tunic formation*)

2 Insects

Of all the invertebrate phyla, insects have arguably received the most attention in ED research because of the commercial interest of insecticide manufacturers. Disruption of the endocrine systems, whether it be by disrupted moulting, growth or reproduction, is an obvious means of incapacitating insects and has therefore been incorporated into a great many pesticides. In addition, the Phylum includes the fruit fly *Drosophila*, one of the most frequently used organisms in genetic studies because of its rapid life cycle.

The Environment Agency 1999 review reported only on the current understanding of ED in aquatic invertebrates; it did not report on terrestrial insects. The main aim of this report is to explore significant advances since the 1999 review, so terrestrial insects will not feature heavily here. Although there are some truly aquatic insects, such as some beetle species, the majority of insects are aquatic only in their early life stages (for example, mayfly nymphs).

2.1 Endocrinology

Compared with crustaceans and other invertebrates, a great many more studies have been published on the endocrinology of insects. Insect hormones control processes such as moulting, yolk synthesis, diuresis, polyphenism, and diapause (Soin and Smagghe, 2007). Peptide hormones are produced from protein pre-cursors in the midgut gland and central nervous system by individual cells (Soin and Smagghe, 2007). Insects cannot synthesise steroids, and instead need to obtain sterols from their diet (Soin and Smagghe, 2007).

Most insect endocrinology research has focussed on ecdysteroids and juvenile hormones (regulating moulting and development, respectively) as these are of commercial interest to the insecticide industry.

Socha *et al.* (2004) showed that different wing-morphs of the firebug, *Pyrrhocoris apterus*, had different amounts of specific proteins present in the accessory glands, and that this was under the control of the juvenile hormone.

A number of studies have explored the hormonal control of ecdysis in insects. A study of *Drosophila* showed that ecdysis is under the control of the ecdysis-triggering hormone (ETH) and eclosion hormone (EH), where these hormones regulate the release of each other via a positive feedback mechanism (Clarke *et al.*, 2004). However, these two hormones alone do not control ecdysis and other neuropeptides are thought to be involved. For example, corazonin (a neuropeptide of widespread occurrence in insects) is considered to play an important role in the initiation of ecdysis (Kim *et al.*, 2004b).

Allotropin is a neuropeptide. This hormone is able to stimulate the production of the juvenile hormone in moths. However, allotropin is multifunctional, being involved in the nervous system and muscular signalling (Elekonich and Horodyski, 2003).

Because a number of pesticides have been developed to specifically target the endocrine system, they can be used experimentally to investigate certain endocrine-mediated responses. For example, methoprene is an insecticide that is a juvenile hormone analogue, that is, it mimics the behaviour of the juvenile hormone.

Zera and Zhao (2004) used the hormone-mimicking behaviour of methoprene to determine whether genetic-based alterations of the endocrine system led to different morphs in insects. The cricket *Gryllus firmus* has two morphs: a long-winged flying form

and a short-winged non-flying form. Following exposure to methoprene, the lipid metabolism of the long-winged morph changed to become more like that of the short-winged form (for example, there was increased oxidation of fatty acids) (Zera and Zhao, 2004). Methoprene exposure also caused increased ovarian growth and a reduction of body fat in the long-winged morph. The extent of changes in the long-winged morph was equal to the normal differences between unexposed long- and short-winged morphs (in other words, the long-winged morphs became phenocopies of the short-winged crickets). Although there is a genetic basis for the lipid metabolism of these two cricket morphs, it is under endocrine regulation (Zera and Bottsford, 2001; Zera and Zhao, 2003).

The juvenile hormone helps control the looping of left-right asymmetric organs in *Drosophila meganolaster* (Adam *et al.*, 2003), whereby excess juvenile hormone leads to incomplete looping of the spermiduct and genitalia. We know that the mechanism by which ecdysis is initiated is conserved in all insect species (Zitnan *et al.*, 2003). Pre-ecdysis triggering hormone (PETH) and ecdysis-triggering hormone (ETH) originate in endocrine Inka cells and act directly on the central nervous system; Inka cells have been found in representatives of all the major insect orders (Zitnan *et al.*, 2003). The number and shape of the Inka cells varies a great deal between species, but they all produce peptide hormones that are released at ecdysis. If extracted, these hormones can trigger ecdysis behaviour when injected into pupae or adult silkworm, by acting on the abdominal ganglia (Zitnan *et al.*, 2002). The Inka extract contains pre-ecdysis triggering hormone (PETH), ecdysis-triggering hormone (ETH) and ETH-associated peptide (ETH-AP).

Contrary to what has been reported for other insects, calcium is not required to stimulate steroidogenesis in the gonads and endocrine cells of the blowfly *Phormia regina* (Maniere *et al.*, 2002). Indeed, these authors report that calcium appears to inhibit steroidogenesis in previtellogenic and vitellogenic ovaries of *P. regina*.

Richard *et al.* (1998, 2001) suggest that juvenile hormone is not essential to the regulation of vitellogenesis in *Drosophila melanogaster*, proposing instead that ecdysteroids are more important. For example, 20-hydroxyecdysone terminates pre-vitellogenic diapause in *D. melanogaster*, but JH III fails to do so (Richard *et al.*, 2001). These authors propose that JH stimulates early yolk protein synthesis by ovarian follicle cells, since it reverses the delay of reproductive development in JH-deficient strains of *D. melanogaster*.

ETH is essential to the initiation of the moulting sequence, since deletion of it causes behavioural and physiological changes in *D. melanogaster* , leading to the death of the fly (Park *et al.*, 2002).

LaFont (2000b) reviewed molecular approaches to ED in insects in 2000, something that was essentially lacking in the literature. Use of molecular tools in ED research is beyond the scope of this report, but some of the contents are presented in the *Horizon scanning* report on genomics (Environment Agency 2007a)

Finally, it has recently been reported that, in addition to the considerable intraspecific differences in sensitivity and response of insects to EDCs, interspecific variability may also exist. Malausa *et al.* (2006) exposed the peach-potato aphid (*Myzus persicae*) to 20-hydroxyecdysone (20E) and then produced clones from these treated aphids. The clones differed significantly in their responses; for example, while some clones avoided food treated with 20E, others preferred it.

2.2 Bioassays and biomarkers

Since the 1999 review, little addition to our knowledge of the endocrine systems of aquatic insects has been made. Instead, the majority of investigations have focussed on the effects of exposure to EDCs. As with the other invertebrate groups, a lot of the experimental work performed since the 1999 review is similar to previous work.

Mosquitoes have received particular attention because of their ability to host and transfer human pathogens. Beckage *et al.* (2004) examined the potencies of three different ecdysone agonist insecticides on the larvae of different mosquito species. Of the three insecticides tested, RH-2485 (methoxyfenozide) was identified as the most toxic, followed by tebufenozide (RH-5992) and RH-5849 respectively. *Aedes aegypti*, *Anopheles gambiae* and *Culex quinquefasciatus* were the test species used, and *A. aegypti* was the most resistant (Beckage *et al.*, 2004). As expected, the mosquitoes initiated the moulting process, but were unable to complete it. Similarly, the pesticide DI-31, a brassinosteroid (phytochemical) analogue that acts as an ecdysone antagonist, prevented *A. aegypti* larva from moulting successfully (Davison *et al.*, 2003). The concentration range for biological effects of DI-31 on *A. aegypti* was narrow, with a no observed effect concentration (NOEC) of 30 µg L^{-1} and an LC50 of 40 µg L^{-1} (over 19 days) (Davison *et al.*, 2003). Sensitivity of *A. aegypti* to DI-31 decreased with moult stage. Halofenizide (another ecdysteroid) is lethal to 4^{th} instars of the mosquito *C. pipiens* at 12.6µg L^{-1} (24h LC50), causing death by preventing the developing instar from moulting (Boudjelida *et al.*, 2005).

The majority of the remaining aquatic insect studies have focussed on the midge larva *Chironomus riparius* (for example, Hahn *et al.*, 2002; Fisher *et al.*, 2003; Watts *et al.*, 2001, 2003). Furthermore, the OECD (2004a, b) and USEPA (2000) have both developed bioassays using chironomids (Taenzler *et al.*, 2007).

Field studies suggest that *C. riparius* develop malformations of their mouthparts (pecten and mentum) when exposed to contaminants. Consequently, deformed mouthparts of *C. riparius* have been used as an ecotoxicological indicator of exposure to contaminants for several years, and continue to be investigated for EDCs. Recently, deformation of mouthparts has been recorded in *C. riparius* following laboratory exposure to chemicals of known ED activity. Mouthpart deformities have been recorded in the larval stages of *C. riparius* following exposure to 10 ng L^{-1} ethinyloestradiol (EE2) and 10 mg L^{-1} bisphenol A (BisA) (Watts *et al.*, 2003); 10 µg L^{-1} 4-nonylphenol (4NP) (Meregalli *et al.*, 2001). However, results are not always conclusive. For example, whereas Watts *et al.* (2003) found mouthpart deformities in larvae exposed to 10 ng L^{-1} EE2, Meregalli and Ollevier (2001) found no deformities in midge larvae exposed to 100 µg L^{-1}. Furthermore, exposure to β-sitosterol failed to induce mouthpart deformities (Vermeulen *et al.*, 2000), and these authors suggested that although deformed mouthparts are usually attributed to ED (in particular the moulting hormone ecdysons), their study did not support such a hypothesis. Finally, it should be remembered that mouthpart deformities can occur in *C. riparius* exposed to compounds other than EDCs, that is, mouthpart deformity is not specifically a symptom of EDC exposure. Therefore in filed surveys, mouthpart deformation could not be used as definitive proof of exposure to EDCs.

Recent studies using *Chironomus riparius* include other common bioassays. Exposure to EE2 and BisA was found to delay moulting in *C. riparius* (Watts *et al.*, 2003). An earlier study by the same authors found that low concentrations of EE2 reduced emergence times (and success) of adult flies from larvae, but that exposure to BisA increased emergence time and decreased the number of adults that emerged successfully (Watts *et al.*, 2001). In contrast, Meregalli and Ollevier (2001) found no significant effect of EE2 on *C. riparius*. Moulting time of *C. riparius* was also investigated by Vermeulen *et al.* (2000) who reported that both lead and mercury

delayed and reduced moulting success; however, similar to their results for mouthpart deformities, the authors found no effect of β-sitosterol. Moulting hormones have been shown to be affected differently, depending on the sex of the organism being exposed. For example, male *C. riparius* larvae increase their rate of ecdysteroid production when exposed to tributyl tin oxide (TBTO), compared with female larvae who reduced their rate of synthesis (Hahn and Schulz, 2002). Correspondingly, the rate of development of the genital marginal disc was increased in male larvae, but reduced in females, suggesting that the whole development cycle of the larvae is affected (Hahn and Schulz, 2002), not just the moulting cycle. The authors point out that these effects are detectable at concentrations 2,000 times lower than lethal effects (LC50), supporting its potential as a bioassay.

As one might suspect, given its potency and effects on other invertebrates, exposure to 4NP (in spiked sediments) reduced emergence of adult *C. riparius* (Bettinetti and Provini, 2002). Sex ratio of emerging flies was unaffected, but emerging chironomids did not lay eggs at concentrations greater than 250 µg 4NP g^{-1} dry weight sediment (the EC10). The authors themselves state that it was not clear whether these impacts were a result of the toxic nature of 4NP, or its activity as an EDC (Bettinetti and Provini, 2002). Hahn *et al.* (2002) reported an inverted dose-response relationship between NP and yolk immunoreactivity (an ELISA-based test for vitellogenesis, where increased immunoreactivity corresponds to increased vitellogenesis). At low concentrations (less than 30 µg L^{-1}) immunoreactivity was reduced, but at high concentrations (2 mg L^{-1}) it was increased, compared with control animals (Hahn *et al.*, 2002). In contrast, BisA exposure (10, 100 and 3,000 µg L^{-1}) led to decreased immunoreactivity (Hahn *et al.*, 2002).

Finally, as with other organisms, induction of the chironomid cytochrome P450 system was measured following exposure to EDCs (Fisher *et al.*, 2002). Exposure of *C. riparius* to pemethrin and phenobarbital (PB) led to induction of the P450 system, as measured by EROD activity (ethoxyresorufin-O-deethylase) (Fisher *et al.*, 2003).

For a more detailed review of the use of chironmids in ED bioassays see Taenzler *et al.* (2007).

Clearly, mosquito and chironomid larvae are the dominant aquatic insects used for ED studies. However, mayfly larvae have received a little attention in this area. *Epeorus latifolium* and *Baetis thermicus* have been used in mesocosm studies (indoor streams) to study the effects of insecticides (Tada and Hatakeyama, 2000). Chronic exposure to fenucarb (1.0 - 2.0 µg L^{-1}) in the experimental streams led to fewer mayfly reaching the third moult, and fewer adults emerging successfully. The authors suggested that this was evidence of ED, although a discrete mechanism/pathway was not demonstrated. Licht *et al.* (2004) exposed *Rhithrogena semicolorata* and *Ephemerella ignite* to fenoxycarb in indoor artificial streams. An LC50 of 3.3µg L-1 was recorded for *R. semicolorata* introduced to the streams 24h before application of the fenoxycarb. 90 per cent of *E. ignite* larvae added to the streams 72 days after application of the fenoxycarb (when the pesticide concentration was below detection limits; 0.5ng L^{-1}) had physical abnormalities when they emerged as adults (Licht *et al.*, 2004).

In 1999, Defur *et al.* (1999) identified a need to find a suitably reliable and robust endpoint for using insects as biomarkers of ED in the field; this remains a research need (Boudjelida *et al.* 2007). Furthermore, there are no published investigations of ED effects on insects in the field. Thus, although some progress has been made in improving our understanding of insect endocrine systems, together with supporting laboratory evidence of EDC effects, the findings of since Environment Agency (1999) are still valid today.

3 Crustaceans

The phylum Crustacea has featured substantially in aquatic toxicity testing and, not surprisingly, a large number of studies have been published on ED in crustaceans. In the Environment Agency's review (1999), three classes of crustaceans were identified as being used in toxicity testing for EDCs: Branchipoda (such as cladocerans), Copepoda and Malacostraca (such as isopods, amphipods and mysids). Representatives of these classes are used routinely by regulatory organisations such as the US EPA (United States Environmental Protection Agency) and ASTM (American Society for Testing and Materials) and, therefore, still feature frequently in the ED literature. However, although further studies have been published since the 1999 review, few advances in the understanding of crustacean endocrinology have been made.

Most endocrinology studies of crustaceans have focussed on the Class Malacostraca (LeBlanc, 2007). As described in the 1999 review, the endocrine system of crustaceans is largely based on neuropeptide signalling, using peptide hormones [such as the crustacean hyperglycaemic hormones (CHHs) and androgenic gland hormone (AGH)]. However, important non-peptide hormones are also found in crustaceans, such as ecdysteroids (involved in moulting and reproduction) and methyl farnesoate (MF) (suspected to be involved in moult regulation, protein metabolism and reproduction). In addition, some vertebrate-type steroids have been found in crustaceans, although a definitive role has not been proven for these hormones. A summary of the main hormones utilised by Crustaceans is presented in Table 3.1

Table 3.1: Type, source and functions of the main hormones used by crustaceans (reproduced from Kusk and Wollenberger, 2007).

Hormone	Biochemical group	Source	Function/controlled process
Ecdysone	Steroid	Y-organ and gonad	Molting, vitellogenesis
Vitellogenesis stimulating ovarian hormone (VSOH)	Steroid (?)	Ovary	Vitellogenesis, female secondary sexual characteristics
Oestradiol, testosterone, progesterone	Vertebrate-type steroids	(Unclear)	(Functional role under debate)
Methyl farnesoate (MF)	Terpenoid	Mandibular organ	Metamorphosis, reproduction, stimulation of molting
Molt-inhibiting hormone (MIH)	Peptide	X-organ/sinus gland	Ecdysteroid synthesis
Gonad- (vitellogenesis-) inhibiting hormone (GIH or VIH)	Peptide	X-organ/sinus gland	Vitellogenesis
Gonad-stimulating hormone (GSH)	Peptide	Protocerebrum	Female and male reproduction
Mandibular organ-inhibiting hormone (MOIH)	Peptide	X-organ/sinus gland	Molting, growth and reproduction
Crustacean hyperglycemic hormone (CHH)	Peptide	X-organ/sinus gland	Energy metabolism, inhibition of molting
Androgenic gland hormone (AGH)	Protein	Androgenic gland	Sexual differentiation

Since 1999, several investigations into the crustacean endocrine system have been published. Most studies have concentrated on ecdysteroids and moulting (see Okumura and Aida, 2000, 2001; LeBlanc 2007), although the role of MF in growth and reproduction has received further attention (see Laufer *et al.*, 2002; Reddy *et al.*, 2004; LeBlanc 2007).

Okumura and co-workers performed a number of studies on hormonal control of the moult cycle of a large freshwater prawn (*Macrobrachium rosenburgii*) (Okumura and Aida, 2000, 2001; Okumura *et al.*, 2003). Early work examined the effects of removal of the eyestalks in the prawn (known to contain hormone secretory centres, namely the X and Y organs and the sinus gland). Although removal of the eyestalks disrupted the duration and timing of the moult cycle, typically by shortening it, the prawns continued to moult (Okumura and Aida, 2001). This result is not unexpected, since removal of the eyestalk removes the source of moult-inhibiting hormone (MIH) (Environment Agency 1999, and references therein). Indeed, removal of the eyestalk can lead to increased concentrations of ecdysteroids in the haemolymph (Laufer *et al.*, 2002). In the same experiment, eyestalk removal disrupted, but did not stop, vitellogenesis (Okumura and Aida, 2001).

An earlier study had recorded 20-hydroxyecdysone (most abundant) and ecdysone present during the moult cycle of *M. rosenburgii*, but concluded that, although present during reproductive moult cycles, these ecdysteroids played no role in ovarian development (Okumura and Aida, 2000). *In vitro* studies of Y-organ secretions confirmed that 20-hydroxyecdysone, 3-dehydroecdysone and high polarity products were secreted by the Y organ, which was shown to control the level of these ecdysteroid hormones in the haemolymph (Okumura *et al.*, 2003). Methyl fernsoate is also known to play a role in moulting and this has been demonstrated by Reddy *et al* (2004), who reported that MF increased the rate of moulting in male and female crabs (*Oziotelphusa senex senex*). Exposure to MF also increased a number of reproductive endpoints (such as egg diameter) (Reddy *et al.*, 2004), and MF is thought to have a controlling influence on allometric growth (Laufer *et al.*, 2002). When MF was administered in low concentrations to spider crabs (in combination with ecdysone) allometric growth was increased, compared with crabs that had been treated with higher concentrations of MF (Laufer *et al.*, 2002). It is assumed that ecdysteroids exert a controlling influence on growth also. Common to most crustaceans, limb re-generation in *Uca pugilator* (a fiddler crab) has been shown to be under the control of retinoid hormones (Hopkins, 2001).

CHH's regulate a range of diverse activities in crustaceans (LeBlanc 2007). For example, moult-inhibiting hormone (MIH) negatively regulates ecdysteroid synthesis by the Y organ and links neurological signalling and steroidal control of moulting (as well as embryo development). Mandibular organ inhibiting hormone (MOIH) negatively regulates the secretion of methyl farnesoate from the mandibular organ and its associated regulatory activities. And gonad inhibiting hormone (GIH), sometimes called vitellogenin inhibiting hormone (VIH) negatively regulates aspects of gonadal maturation (see LeBlanc, 2007).

AGH is produced by the androgenic gland, which has only been identified in Decapods to date, although AGH has been positively identified in other crustacean Orders (LeBlanc, 2007). AGH is responsible for sexual differentiation of males (i.e. mascularization) (LeBlanc, 2007), for example mascularization of the pleopods, development of the male gonophore complexes and conversion of ovarian tissues to testicular tissue. AGH also negatively regulates Vtg production (LeBlanc *et al.*, 1999; LeBlanc 2007). Although AGH is responsible for sexual differentiation, it plays no role in sexual determination (LeBlanc, 2007).

MF is the main terpenoid hormone in crustaceans, and has been measured in more than 30 species (LeBlanc, 2007). MF is suspected to be involved in moult regulation, protein metabolism and reproduction. However, a number of substances are known to act as MF mimics. For example, insecticides based on insect growth regulators (e.g. fenoxycarb) mimic MF, and decapods exposed to these insecticides show delayed or abnormal metamorphosis (e.g. Cripe *et al.,* 2003).

No definitive role has been proven for vertebrate-type steroids hormones in crustaceans.

A limited number of advances have been made in the area of biochemistry/molecular biology with regard to the endocrine system of crustaceans. For example, Maibeche-Coisne *et al.* (2001) isolated and characterised an aldoketoreductase enzyme (AKR) from the crayfish *Orconectes limosus.* The transcript was shown to have restricted expression in the antennal gland, which was constant throughout the moult cycle; in contrast, expression in the labyrinth was strong (Maibeche-Coisne *et al.* 2001). Rewitz *et al.* (2003) reported sequences for two cytochrome P450 (CYP) enzymes from the shore crab, *Carcinus maenas* (CYP330Al and CYP4C39). CYP enzymes catalyse the metabolism of endogenous (and exogenous) substances, including hormones. Two hormones, ecdysone and ponasterone, induced the expression of CYP330Al in male intermoult crabs; this did not occur in red crabs, suggesting that CYP330Al is involved with ecdysteroid catabolism. However, exposure to xenobiotics (benzo(a)pyrene (BaP) and phenobarbital) caused similar expression of CYP330Al, indicating an interaction between endocrine function and chemical exposure (Rewitz *et al.*, 2003). Expression of the CYP4C39 enzyme was unaffected by ecdysteroids (or pollutants). Nitric oxide synthase (NOS), in addition to being used for neuromodulation, is now thought to be involved in modulating the synthesis of ecdysteroids in decapods, because NOS mRNA has been found in the Y organ (Kim *et al.*, 2004a). Biochemical aspects of the CHH's are reported by Chen *et al.* (2005).

In summary, some progress has been made in our understanding of the endocrine system of crustaceans, but advances have been small and not on the scale recommended by the Environment Agency review (1999).

3.1 Bioassays and biomarkers

Because of their use in ecotoxicological testing, and their adoption as standard test organisms by regulatory authorities such as the US EPA, and other international bodies such as the OECD, ,some crustacean species have received more attention with regard to ED research than others. Cladocerans (such as *Daphnia magna*), copepods (such as *Acartia tonsa*), amphipods (such as *Corophium volutator* and *Gammarus* sp.) and mysids (such as *Americamysis bahia*) remain key testing species; most ED research in crustaceans has, unsurprisingly, focussed on these classes/orders.

Endpoints already used in ecotoxicological testing that might also be suitable for identifying EDC exposure and effects include: larval development, growth, moulting, energy metabolism, steroid metabolism, reproduction, vitellogenesis, morphology/histology, and life-cycle (DeFur *et al.*, 1999; Environment Agency, 1999; Verslycke *et al.*, 2002; Ghekiere *et al.*, 2007). Of course, many of these processes are inter-related; for example, growth can only occur as a result of moulting, and fecundity is directly related to female size. Thus, disruption of one process has potential knock-on effects on other processes.

Bioassays suggested for use in identifying ED effects must be evaluated carefully. A common problem is that some suggested endpoints are also affected by exposure to anthropogenic substances other than EDCs; this may be complicated further by

interactive effects of chemicals and/or responses. Further discussion of the use of crustacean biomarkers for ED is presented in LeBlanc (2007).

3.1.1 Copepods

To date, most ED research in copepods has focussed on the use of life-cycle or development assays; and these have had mixed success in identifying ED effects (see Anderson *et al.*, 2001; Marcial *et al.*, 2003). For example, in the harpacticoid *Tigriopus japonicus*, exposure to a number of oestrogenic compounds (natural and anthropogenic) increased the number of days it took nauplii to reach the copepodite stage and then sexual maturity; these effects carried over to the next generation (Marcial *et al.*, 2003). Wollenburger *et al.* (2005) also demonstrated disruption of development due to exposure to brominated flame retardants (BFRs). *Acartia tonsa* nauplii exposed to tetrabromobisphenol A (TBBPA), tribromophenol (TBP), and four polybrominated diphenyl ethers ([PBDEs]: BDE-28, BDE-47, BDE-99, and BDE-100) also showed significantly inhibited development compared with controls. BFRs were shown to have ecdysteroid antagonist properties (Wollenberger *et al.*, 2005). But in *Nitocra spinipes* (another harpacticoid) exposed to anthropogenic oestrogens, naupliar development was unaffected (Breitholtz and Bengtsson, 2001). And in the marine copepod *Tisbe battagliai*, life-cycle tests following exposure to ecdysteroid agonists (such as 20-E), oestrogen agonists (such as diethylstilbestrol (DES), 17beta-oestradiol, oestrone and 17alpha-ethinyloestradiol) and a pharmaceutical anti-oestrogen (ZM189, 154), showed significant toxicity only for 20-E and DES (Hutchinson, 2002). More recently, Forget-Leray *et al.* (2005), in response to concern that STW effluents contained EDCs, exposed the commonly tested estuarine copepod *Eurytemora affinis* to a number of EDCs potentially present in STW effluents (substance tested were oestradiol (E2), BaP, 4NP, di(ethyl-hexyl)phthalate (DEHP), and atrazine (A)). Effects on adults (reproductive output) and nauplii (development) were investigated and NOECs produced for each response/chemical. The authors found that this species was affected by exposure to EDCs, especially when exposed to the substance as nauplii (Forget-Leray *et al.*, 2005).

Perhaps more useful for identifying whether ED is occurring in crustaceans is the ELISA test recently described for quantifying vitellin (VTN) in the sediment-dwelling copepod *Amphiascus tenuiremis* (Volz and Chandler, 2004). The assay uses a VTN-specific polyclonal antibody (developed against an amphipod) that is specific to female copepod proteins, is capable of detecting (and measuring) VTN at 2 ng ml^{-1} and can discriminate between gravid female and male copepods (Volz and Chandler, 2004). When tested experimentally against an EDC (the insecticide fiprinol), the assay showed virgin females to have elevated VTN compared with control females; males were unaffected.

The widespread use of copepods in laboratory toxicity testing for the aquatic environment is reflected by their inclusion in the OECD's list of test species. Over the last few years the OECD has developed guidance on a Copepod Development and Reproduction Test. Various drafts have been produced, the most recent, reporting validation via an international ring-test, was completed at the end of 2006 (OECD, 2006). Endpoints in the OECD recommended test for calanoid copepods include: hatching success, larval development ratio (LDR) and survival of early life stages for F_0 and F_1 generations, fecundity and body length. Harpacticoid copepod endpoints include: rate of development to copepodite stage, survival of early life stages, rate of development to adult stage, fertilization success, viable offspring production and time to production of first clutch (Kusk and Wollenberger, 2007)

3.1.2 Amphipods

Amphipods have also received much research effort with regards to ED over the last five years and, as with other crustaceans, reports of assays using development as an endpoint are common. However, more than for any other group, investigations into the role of parasites in the natural disruption of sex ratio are also numerous (for example, Ford *et al.*, 2004a; Ford *et al.*, 2004b; Rodgers-Gray *et al.*, 2004).

Life-cycle or development assays for amphipods follow the same general practice as those reported for other crustaceans (see mysids and copepods). For example, Ohji *et al.* (2003) exposed *Caprella danilevskii* embryos to between 10 and 1,000 µg TBT-Cl L^{-1}, and reported that the population became increasingly dominated by females with increasing exposure to TBT-Cl (up to 86 per cent female at 1,000 µg L^{-1}). Other reproductive outputs were also affected by TBT-Cl (such as reduced brood size). TBT-Cl was reported to inhibit reproduction in *Hyallela azteca*; however, results were extremely variable (Bartlett *et al.*, 2004). Potential effects of TBT are well known from studies with dogwhelks, so it is not that surprising that TBT also causes ED in crustaceans.

Physical development of sexual appendages and organs is also affected by EDCs, and may be a potential indicator of ED exposure in natural populations. Vandenburgh *et al.* (2003) reported that second generation male *Hyallela azteca* developed smaller gnathopods, and had morphological abnormalities of the reproductive tract following exposure to 0.1 µg EE2 L^{-1}. Indeed, some males demonstrated characteristics consistent with hermaphroditism. Similarly, Gross *et al.* (2001) reported alterations of gnathopod (and genital papillae) size in a population of *Gammarus pulex* inhabiting a site that received STW effluent. Additionally, effluent-exposed amphipods were significantly shorter, with males being more similar in size to female (Gross *et al.*, 2001). Gnathopod size is generally accepted to be under the control of the androgenic gland (Ford *et al.*, 2004a) and appears to be consistently affected in amphipods exposed to EDCs; it may prove to be a useful biomarker of ED.

Intersexuality is not uncommon in amphipods and can occur for reasons other than exposure to ED (see Ford *et al.*, 2004b, Jungmann *et al.*, 2004, Rodgers-Gray *et al.*, 2004). The simple occurrence of intersex in a population is not a feasible biomarker of EDC exposure. For example, in *Gammarus duebeni* the parasite *Nosema granulosis* (a microsporidean) can alter males to the extent that they become reproductively functioning females (Rodgers-Gray *et al.*, 2004). The parasite is thought to block differentiation of the androgenic gland, thereby preventing androgenic hormones being produced and thus preventing differentiation into males.

Although there are increasing concerns of ED in invertebrates, few studies have explored the implications of intersex on the population as a whole. Ford *et al.* (2004a) reported some useful information on the physical costs of intersexuality in the marine amphipod *Echinogammarus marinus*: intersex *E. marinus* have reduced fertility and fecundity, and attain a larger size at sexual maturation (hypothesized to lead to reduced pairing success).

3.1.3 Cladocerans

A number of studies have reported on different chemicals affecting *Daphnia* sp, the large number of studies presumably attributable to *Daphnia*'s widespread use in ecotoxicological testing (see Tatarazako and Oda, 2007). In addition, the OECD has developed a reproduction test using Daphnia magna for detecting effects of EDCs (OECD, 1998).

As with other crustaceans, the most common endpoints measured, especially for EDCs, are reproductive output and development. In *Daphnia magna*, the development of secondary sexual characteristics (such as first antennae of males and abdominal process of females) was affected by exposure to the juvenile hormone analogue (JHA) pesticide methoprene at 0.08 µg L^{-1} and exposure to androstenedione (a vertebrate steroid androgen) at 6 µM (Olmstead and LeBlanc, 2000). Indeed, methoprene has been shown to illicit a number of hormone-controlled effects in *D. magna* due to both agonism and antagonism of some juvenoid receptors (Olmstead and LeBlanc, 2001). Furthermore, having demonstrated that the crustacean juvenoid hormone methyl farnesoate programs oocytes of the crustacean *D. magna* to develop into males, the same research group demonstrated that another JHA (pyriproxyfen) would mimic methyl farnesoate, causing a male-dominated brood (Olmstead and LeBlanc, 2003). In contrast, the pesticide dieldrin (known to act as an oestrogen mimic) reduced the production of males in *D. galeata mendotae* (Dodson *et al.*, 1999). Styrene is reported to reduce reproductive output in *Ceriodaphnia dubia* (Tatarazako *et al.*, 2002), and the pesticide toxaphene increases the production of males in *D. magna,* although the mixed function oxidase (MFO) system is reported to mediate this response (Kashian, 2004).

As a biomarker, incomplete ecdysis has been suggested as an indicator of ecdysteroid exposure in *D. magna* (Baldwin *et al.*, 2001). Although neither 20-E nor ponasterone (PoA) significantly affected moulting frequency, incomplete ecdysis did occur leading to premature death (Baldwin *et al.*, 2001).

It is noted by many authors that developing bioassays to demonstrate ED is difficult as it is often impossible to separate the general toxicity effects of an EDC from its effects that specifically target the endocrine system. Tatarazako and Oda (2007) argue that *D. magna* is especially suitable as a screening bioassay species for juvenile hormone mimics because it reproduces by cyclical parthogenesis, where offspring sex-ratio can be used as a specific ED endpoint.

3.1.4 Mysids

One of the main crustaceans orders to be recognised as potential indicators of ED is the Mysidacea (Crustacea: Peracarida) (Verslycke *et al.*, 2002; 2007). Mysids are already used by a number of regulatory agencies for ecotoxicological testing (see US EPA, 1995; Roast *et al.*, 1998; US EPA, 2002; Verslycke *et al.,* 2004), and wild populations have been shown to be good bioindicators of exposure to a number of anthropogenic substances (see Verslycke, 2003). Since mysids are already used by regulatory agencies, and because they are susceptible to the influences of EDCs, the use of mysids in the risk assessment of EDCs is likely to increase (CSTEE, 1999; DeFur *et al.*, 1999; Verslycke *et al.*, 2002). Indeed, when the US EPA established the Endocrine Disruptor Screening and Testing Standardization and Validation Task Force to co-ordinate scientific and technical work to validate the assays suggested by the Endocrine Disruptor Screening and Testing Committee (EDSTC), mysids were recommended for the *in vivo* testing required at Tier 2 (Verslycke *et al.*, 2004). *Americamysis bahia* is now the only invertebrate species used by the EDSTC (Ghekiere *et al.*, 2007). However, as is the case with most invertebrate species, very little is known about the specific endocrine system of mysids; as with most crustaceans, our limited understanding is drawn from studies with decapods.

A novel assay has recently been described by Ghekiere *et al.* (2007), whereby hatching success is measured by exposing mysid embryos to EDCs *in vitro*. Embryos removed from gravid females are exposed to an EDC in cell plates and a number of parameters are measured as the embryos hatch and then grow; the test terminates at the stage III moult (when the juvenile becomes free-swimming). In their experiments,

Ghekiere *et al.* (2007) used methoprene (a JHA) as a test substance, which reduced hatching success and lowered survival at concentrations greater than 1 µg L^{-1}. Detailed analysis of the results demonstrated that although total development time (from fertilisation to the stage III moult) was unaltered by methoprene exposure, the length of the stage II phase was extended. Why this occurred, and what (if any) the consequences are of an extended phase II and shortened phase III development, is not clear. This assay does not demonstrate that ED is happening, although knowing the mode of action of the test substance leads one to assume that it is. However, it is of course possible that other, non-ED substances might also impact on development time.

Moulting, as in other crustaceans (Environment Agency, 1999), is affected by ecysteroids under the immediate control of the Y organ (Verslycke *et al.*, 2002). In arthropods, the ecdysone receptor is in the same gene family as the thyroid gland in vertebrates, but the receptor is unaffected by steroids. However, some non-steroidal EDCs have been shown to be ecdysone receptor antagonists (lindane, BisA, and *p,p'-DDT*).

The MFO system is known to be responsible for the metabolism of organic toxicants, but it is also responsible for steroid metabolism (Verslycke *et al.*, 2002). Consequently, if the MFO system is induced by toxicants, this may have knock-on effects on the hormonal control of reproduction, growth and so on. Furthermore, it has been suggested that endogenous androgens might be precursors to other hormones. If this is true, then exposure to exogenous androgens (or mimics) may trigger other hormone receptors in addition to the androgen receptor. To date, there is little evidence to support this hypothesis, although Verslycke *et al.* (2002) reported the sex-specific production of androgens (such as testosterone and androstenedione) in *N. integer.* Certainly, steroid metabolism can be affected by exposure to contaminants of known ED effects. For example, exposure to TBT-Cl affected testosterone metabolism in *N. integer* (as measured by analysis of metabolites): reductase activity and metabolic androgenization were induced in the 10 ng L^{-1} treatment, whereas higher concentrations resulted in a reduction of sulfate conjugation (Verslycke *et al.*, 2003).

The MFO system has received much attention in recent years for its potential as a biomarker of toxicant exposure, and for identifying the effects of such exposure. It seems likely that research in this area will increase in the area of EDCs. However, due to the complexity of the system and its general role in the metabolism of toxicants, it is unclear whether ED can be specifically identified. Verslycke *et al.* (2002) outlined several ways forward in the study of the MFO system - these are currently being studied in *A. bahia* (Verslycke, personal communication)

Perhaps one of the most promising areas of EDC research in crustaceans is the development of accurate assays for the detection of vitellin (see Section 3.2.1), and this area of research is being explored in mysids (see Tuberty *et al.*, 2000; Ghekiere *et al.*, 2004). A quantitative enzyme-linked immunosorbent assay (ELISA) has been developed for *A. bahia* (Tuberty *et al.*, 2000), and Ghekiere *et al.* (2004) have purified and characterised vitellin in *N. integer* using gel filtration and electrophoresis respectively. Specific polyclonal antibodies for *N. integer* vitellin have been produced and are currently being used to develop immunoassays to study vitellogenisis in this mysid (Ghekiere *et al.,* 2004; personal communication). These assays might then be able to identify ED disruption of vitellogenesis. Clearly, this research might provide useful tools for studying ED effects in mysids; however, further work is currently required to assess the suitability of vitellin assays for measuring ED.

Female mysids carry their developing embryos/juveniles in a ventral brood-pouch or marsupium (giving rise to their common name of 'opossum shrimps'). This characteristic makes mysids well suited to life-cycle studies, and the standard *A. bahia* life-cycle assay developed by the US EPA can be modified for any mysid species. Transgenerational responses have been reported in *A. bahia* following exposure to

fenoxycarb (a pestcide that acts as a juvenile hormone agonist) (McKenney *et al.*, 1999). Although first generation juveniles showed reduced growth, reproductive output was not significantly affected. However, second generation adults (which had been exposed as embryos in the marsupium to fenoxycarb) did have reduced reproductive output. ED, however, was not specifically identified.

Although the review of Verslycke *et al.* (2004) discusses the potential of mysids as test organisms for evaluating the effects of EDCs, it is apparent that, with few exceptions, assays for identifying ED have not yet been developed. Mysids are undoubtedly sensitive to toxicants and ecologically important; given their use in standard ecotoxicological testing by the US EPA, they are likely to be used in ED studies (Verslycke *et al.*, 2004). Many of the assays developed to date, however, do not show ED specifically, and this remains a research need. Areas where endpoints might show definitive ED effects include the MFO system and immunoassays to study vitellogenesis.

3.1.5 Other crustaceans

In addition to the routinely used species described above, other crustaceans have received attention in ED research, including decapods (crabs and shrimp), cirripedes (barnacles) and anostracans (brine shrimp). In green shrimp (*Neocaridina denticulata*), the production of vitellogenin-like protein was induced following exposure to 10 ng L^{-1} of the oestrogen mimic chlordane (Huang *et al.*, 2004; Huang and Chen, 2004). In the same study, oestrogen levels were increased and testosterone levels reduced following exposure to chlordane and lindane. Vitellin from grass shrimp (*Paleamonetes pugio*) was purified and monoclonal antibodies raised for the detection of lipovitellin proteins (Oberdorster *et al.*, 2000). In contrast to previous studies, cross-reactivity of the antibodies was found with blue crab (*Callinectes sapidus*), mud crab (*Rhithropanopeus harrisii*) and red crayfish (*Procambarus clarkii*). Grass shrimp are often used in standard ecotoxicology tests, and have been used in life-cycle tests to demonstrate pesticide effects (including pesticides whose mode of action is ED). For example, embryonic development and metamorphosis success of *P. pugio* is inhibited following exposure to fenoxycarb (McKenney *et al.*, 2004).

3.2 Conclusions

Numerous laboratory studies have shown that chemicals with known ED properties affect crustaceans. Various bioassays, developed and standardised for ecotoxicological testing of a wide range of substances, demonstrate that EDCs can impact on non-target organisms. Endpoints reported include traditional regulatory endpoints such as LC50s, and new ED-specific endpoints such as vitellin induction. Between these extremes, there are a number of endpoints which, although clearly affected by EDCs, may or may not be attributable specifically to ED. Additionally, there are some potentially useful assays (such as embryonic development) that show no significant effects even though the test substance may be known to have ED properties.

Since 1999, the most significant advance of ED research in crustaceans appears to be the development of new ED-specific tests. Vitellin assays show good potential, although further work is required. The involvement of the MFO system is another area that could provide useful, ED-specific information. A number of development and life-cycle assays, although not necessarily ED-specific, seem able to provide information on potential population effects via impacts on reproductive output. Although there has been some progress since 1999, further work in these areas remains a research need.

4 Molluscs

Whereas ED of insects has been researched a great deal for economic reasons (for example, for insecticide development), ED of molluscs has received attention because of severe adverse affects found in the natural environment. Imposex (the superimposition of non-functioning male genital tracts, such as a penis and vas deferens, on females) in gastropods (primarily the dogwhelk *Nucella lapillus*) following exposure to TBT is arguably the best known single case of ED reported to date.

Effects of organotins (bi- and tributyl tin, and bi- and triphenyl tin) are still widely investigated in gastropod molluscs; more than 100 research papers have been published in this area alone since 1999. However, in this instance, the number of papers does not necessarily represent the number of new or significant advances in molluscan ED. TBT was an additive used worldwide in maritime paints because of its potent antifouling properties. The ED effects of TBT in gastropods were first discovered in the 1980s (see Bryan *et al.*, 1986) and because of this, the use of TBT-based paints on sailing vessels smaller than 25 metres was banned in Europe. However, TBT effects are still being reported, and occurrence of imposex in different geographical locations or gastropod species remains the subject of much of the literature (for example, Strand and Jacobsen, 2002; Marshall and Rajkumar, 2003). Furthermore, because the TBT-imposex link is so well documented, gastropods are often used as biomarkers for TBT (see Quintela *et al.*, 2000; Birchenough *et al.*, 2002; Fernandez *et al.*, 2005). Effects of TBT, TPT and so on have been well documented for the last 20 years, and are not considered appropriate for inclusion in this review. Research efforts in this area are acknowledged but not reviewed here. A description of possible mechanisms for the effects of TBT in gastropods is presented in Appendix 2.

The following sections are taken from a report by Rachel Benstead (Environment Agency, Biological Effects Laboratory) presenting some recent information on ED and gastropods.

4.1 Endocrinology

In vertebrates, the peptide hormones (gonadotrophin-releasing hormone (GRH) and gonadotrophin) are secreted by the hypothalamus and pituitary (respectively) to trigger the gonads to synthesis sex steroids, leading to the production of gametes (LaFont, 2000a). Although the endocrine system of gastropods is far simpler than that of vertebrates, peptide hormones are still produced (by ganglia) to trigger and control gamete production. Gonadotrophin controls gonad maturation, and the abdominal ganglia produce egg-laying hormone (ELH; another neuropeptide produced in abdominal ganglia) which controls egg mass production and egg-laying behaviour. Essentially, molluscs use 'true' hormones for chemical signalling within their body tissues, including the gonadotrophic hormone and vertebrate-type sex steroids, which they are able to produce *de novo* in the gonad.

Unsurprisingly, much of the research on endocrines systems (and potential ED) of molluscs has focussed on marine bivalves such as oysters (for example, *Crassostrea sp.*), mussels (such as *Mytilus sp.*) and clams (for example, *Mya sp.*) because they are commercial crop organisms. Within the Bivalvia, there is some evidence for steroids being manufactured and used in biochemical pathways; however, further work is needed to understand their role in the bivalve life cycle. Much research has focussed on protecting valuable shell-fisheries from the effects of ED pollutants, or developing ways to increase spat production, and so on. There is evidence to suggest that steroid hormones and their analogues are involved in the reproductive process, but as yet

there is no definitive evidence that exposure to steroids can cause detrimental effects on the ability of individuals to reproduce.

Blaise *et al.* (1999) examined the haemolymph from the bivalve *Mya arenaria* for the presence of proteins similar to (and providing the same function as) the egg-yolk precursor vitellogenin. An alkali-labile phosphate assay (ALP assay) correlated with a trout immunoassay for vitellogenin, indicating that vitellogenin-like (Vtg-like) proteins were present. Vitellogenin is a glycolipophosphoprotein that contains one zinc atom and two calcium atoms per molecule; calcium and zinc concentrations also correlated with the presence of Vtg proteins. ALP levels in clams collected from the wild increased with gonadal maturation and the onset of spawning (Blaise *et al.*, 1999); and haemolymph ALP levels in female mussels varied according to the stage of reproductive cycle, being higher in ripe females than post-spawning females (Gagne *et al.*, 2001; 2001b).

It is possible that steroid receptors in molluscs are not able to distinguish between vertebrate steroids, because although they have different actions they are very similar in molecular structure. To test this, molluscan steroid receptors need to be identified and used in competitive binding assays with the steroids of interest. Work in this area has recently begun, with oestrogen-like receptor proteins being sequenced for both *Marissa cornuarietis* (Bannister, personal communication) and *Aplysia californica* (Thornton *et al.*, 2003). So far, specific binding of any vertebrate steroid has not been achieved for either species. In *A. californica* this protein activates luciferase from a transfected expression plasmid containing a characteristic oestrogen-response element in a cell-based reporter gene assay, suggesting that it may be a constitutive activator of gene expression via the steroid response (Thornton *et al.*, 2003). Research into an oestrogen-related receptor is progressing in *M. cornuarietis* (Bannister *et al.*, personal communication), with the aim of studying the expression of the receptor and the oestrogen receptor-like protein during different stages of the life history.

Research is progressing in identifying steroidogenesis and steroid receptors in bivalves. Following incubation of oyster (*Crassostrea gigas*) homogenates with androstenedione, a steroid precursor, 12 metabolites were identified by thin-layer chromatography, of which two were representative of oestradiol and oestrone (Le Curieux-Belfond *et al.*, 2001). When repeated with the addition of 4-androsten-4-ol-3,17-dione (4OHA) and micronazole (known steroidal and non-steroidal aromatase inhibitors, respectively), activity was inhibited and identified to be enzyme-like (Le Curieux-Belfond *et al.*, 2001). Recently, Janer *et al.* (2004) demonstrated these aromatisation abilities *in vivo* for bivalves. When the mussel *Mytilus galloprovincialis* was exposed to oestradiol, dose-dependent increases were recorded in the body burden of oestradiol, compared with no change in testosterone concentrations. P450 aromatase decreased activity, but acyl-CoA:oestradiol acyltransferase activity increased, suggesting that (a) *M. galloprovincialis* is able to downregulate aromatisation in the presence of sufficient oestrogen, and (b) that *M. galloprovincialis* can deactivate surplus hormones by making them non-bioavailable (Janer *et al.*, 2004). These findings, and the fact that the transformation agent 17β-hydroxysteroid dehydrogenase rises in oysters as sexual maturation proceeds, support the suggestion that bivalves can synthesise steroids.

When exposed to steroid hormones (oestradiol, progesterone and testosterone), male scallops (*Placopecten megellanicus*) increased sperm release 200 to 300 per cent (Wang and Croll, 2003). Egg release also increased. Sperm and egg increase was greatest at lower concentrations (10^{-6} to 10^{-7} M), showing inhibition with increasing concentrations (Wang and Croll, 2003). When antagonists were added with these steroids (tamoxifen, flutamide and RU486 antagonise oestradiol, testosterone and progesterone respectively), effects varied. Only tamoxifen prevented the increase in egg release caused by oestradiol and progesterone exposure; and the stimulatory effect of testosterone on sperm release was blocked by all three antagonists (Wang

and Croll, 2003). Wang and Croll (2004) have also demonstrated that these three steroids can affect gender determination, whereby exposure to testosterone, oestradiol and progesterone changed the male-biased sex ratio of 1.22 to a female dominant ratio of 6.0 to 8.7. The fact that steroids can affect gamete release and gender determination, but their effect can be blocked by analogues, is further evidence for the presence of steroid receptors. However, since all three hormones affect sex ratio, it appears that the receptors are not hormone-specific (Wang and Croll, 2003; Wang and Croll, 2004).

It is a peculiarity that molluscs appear able to use true hormones, and yet the arthropods, a phylogenetically higher order that makes greater use of steroids, seem to lack the enzymes to biosynthesise these sex steroids from the cholesterol precursor. It is for this reason that molluscs are considered more likely candidates than arthropods to replace vertebrates when testing for any endocrine-modulated effects on reproductive systems. This is an important point, particularly for this report, as there is a need to minimise the use of vertebrates in biological testing. Present research, such as that of Bannister and co-workers, will aid in the understanding of the mechanisms of action of vertebrate steroids in gastropods. In turn, this will add further support to the use of these organisms as sentinels of related sub-lethal effects.

4.2 Bioassays and biomarkers

4.2.1 Bivalves

Gagne *et al.* (2004) searched for ED effects in mussels held long-term downstream of a sewage treatment works, by measuring concentrations of metallothionein-like compounds, as well as seratonin and dopamine concentrations. In fish, metallothionein concentrations fall during vitellogenesis; seratonin levels rise and dopamine levels fall during spawning, while the opposite occurs during gametogenesis (Gagne *et al.*, 2004). Mussels held downstream of the outfall had elevated metallothionein-like proteins, suggesting that the mussels needed to detoxify metals from the effluent. Seratonin and dopamine were both reduced, but where the dopamine transport system was uninhibited, the seratonin pathway was not being cleared effectively. Further work is required to establish whether spawning activity is affected, but if it is, this suggests that the STW effluent acts as a neuro-endocrine disrupter of reproductive function.

The same research group reported that ALP levels increased in male and female zebra mussels (*Dreissena polymorpha*) deployed downstream of STW works, compared with laboratory-held control mussels (Quinn *et al.*, 2004). Similarly, ALP levels increased following oestradiol and nonylphenol injections into the adductor muscle of *Mya arenaria* (with a 48-hour incubation period); effect thresholds were in the order of 10^{-2} ηmole (Blaise *et al.*, 1999). With the zebra mussels, histology of STW effluent-exposed mussels showed that while female gonads were unaffected compared with controls, males had significantly more interstitial between the semeniferous tubules, indicating spermatogenesis. The results suggest potential ED, but the exposed mussels also showed reduced condition factors and symptoms of a general toxicological response (Quinn *et al.*, 2004). So although ALP levels do appear to be associated with known EDCs and STW effluents, the results of Quinn *et al.* (2004) are not conclusive of ED, and may be due to other non-ED stressors. If further work demonstrates that ALP does not react to other stimuli, it may prove a useful biomarker for exposure to environmental oestrogens.

Vitellogenin concentration is perhaps a more obvious measurement endpoint for ED, and this has been investigated in bivalves. However, when *Mytilus edulis* and

Anodonta cygnea (marine and freshwater mussel species, respectively) were exposed to 2 mg L^{-1} oestradiol (via aquatic exposure and injection), vitellogenin was not found using gel electrophoresis techniques (Riffeser and Hock, 2002). Fish controls (tench and carp) did produce produce vitellogenin. Only glycoproteins associated with metal binding were produced by the mussels, leading to the assumption that vitellogenin is contained within mussel oocytes and not the haemolymph (Riffeser and Hock, 2002).

Gagne *et al.* (2001a) injected coprostanol into the freshwater mussel (*Elliptio complanata*) to compare its competitive affinity to bind to gonad homogenate with other steroids, and determine whether the homogenate could synthesise steroids. The mussels injected with coprostanol had higher levels of Vtg-like proteins, but not as high as those injected with oestradiol. Similarly, coprostanol did bind to the gonad cytosol fraction, but not to the same extent as oestradiol. High performance thin-layer chromatography (HPLC) analysis of the metabolites showed two compounds similar in polarity to the steroid intermediates androstane and dihydroxycholesterol (Gagne *et al.*, 2001a). Binding saturation of oestradiol in the gonad cytosol occurred 50 ρmol mg total $protein^{-1}$ for both males and females, and was specific to oestradiol (Gagne *et al.*, 2001a).

More concerning in terms of population level effects are the reults of Nice *et al.* (2003). They exposed seven-day old embryos of *Crassostrea gigas* to concentrations lower than 100 ng^{-1} NP for 48 hours, grew the spat on to sexual maturity and then crossed control and treated adults (using males and females from both groups). Embryo survival to 48 hours post-hatch was significantly greater between control males and females than between any cross where one or both parents had been treated with NP, but there was no clear evidence that this was due to endocrine disruption (Nice *et al.*, 2003). Oysters can adopt either gender each year at the start of the sexual maturation cycle, and usually develop a greater proportion of males in yearling cohorts, possibly because spermatogenesis is less energetically demanding than oogenesis. Therefore, if the population is compromised by a stressor more males might be expected, but because females dominated the exposed groups in these experiments, it may be hypothesised that the effect was due to endocrine disruption.

4.2.2 Gastropods

Prosobranchs

Most research on ED in gastropods has focussed on impacts on egg-laying, and much work has focussed on *Marissa cornuarietis*. Following a four-month exposure to 150 ng L^{-1} triphenyl tin (TPT), there was almost a complete cessation in egg-mass production, and those egg masses that were produced contained fewer eggs (Schulte-Oehlmann *et al.*, 2000). Penis length also increased in males and females. In the same study, TPT did not cause intersex in *Nuccella lapillus*, nor affect oogenesis in either species. TPT effects are mostly similar to TBT, and so are not reported in detail here. However, other effects include sterilisation of male *M. cornuarietis*; ovarian changes in *Hinia reticulata* (such as slower maturation of oogonia and blocked oviducts) and degeneration of sperm cells and disruption of germ-cell formation (Schulte-Oehlmann *et al.*, 2000).

Exposure to octyl phenol (OP) and BisA did not affect spermatogenesis or oogenesis in *M. cornuarietis* or *N. lapillus*, but neither species had increased egg production (up to four-fold at 1 µg L^{-1}) (Oehlmann *et al.*, 2000). In *M. cornuarietis* malformation of the oviduct also occurred, including second vaginas, enlarged pallial accessory sex glands and rupture of the duct due to blocked egg masses. *N. lapillus* did not show these

effects, possibly due to its reproductive condition at the time of the study (Oehlmann, personal communication).

Anti-androgens are also reported to affect gastropods. Cyproterone acetate and vinclozolin exposure led to a reduction in the number of male *N. lapillus* with sperm-filled seminal vesicles, suggesting the onset of sexual repose (Tillman *et al.*, 2001). Furthemore, vinclozolin also led to reduced penis length in males, potentially impacting reproductive ability. Additionally, *M. cornuaretis* eggs were exposed to 1 µg L^{-1} of either EE2 or methyl testosterone for nine months, with and without cyproterone acetate. Although imposex occurred in both hormone exposures, the anti-androgen only suppressed the effect in those animals exposed to the oestrogen (Tillman *et al.*, 2001). The oestrogenic imposex effect was thought to be androgen-mediated (caused by a feedback mechanism acting on an aromatase that reduces the conversion of testosterone to oestradiol because enough oestrogen is present) (Tillman *et al.*, 2001).

Given the requirement to reduce vertebrate testing, and the potential suitability of molluscs as testing or biomarker organisms, Jobling *et al.* (2004) compared the responses of a freshwater prosobranch gastropod (*Potamopyrgus antipodarum*) to those of fish when exposed to steroid mimics in the laboratory and to sewage effluents in mesocosm conditions. Twenty-one day laboratory exposures of *P. antipodarum* to EE2 (up to 0.1 µg L^{-1}) caused significant increases in embryos found in the brood pouch [1]. A time-dependent dose-response curve was recorded with a peak response at around 25 ηg^{-1} after nine weeks. A similar effect was seen in the sewage effluent, where exposure to 12.5 per cent and 25 per cent effluent (diluted with clean[1] river water) caused a significant induction of embryo production after 14 days compared with a tap water control; 50 per cent and 100 per cent effluent demonstrated inhibition (Jobling *et al.*, 2004). Results were compared with a recrudescence assay using fathead minnows (*Pimephales promelas*) (see Jobling *et al.*, 2004 for details). As with the gastropod, fish egg production increased with a concentration-dependent response, peaking at 1 ng L^{-1} and with a complete cessation of spawning at 100 ng L^{-1} (Jobling *et al.*, 2004). These results suggest that the effects of STW effluent are similar in fish and *P. antipodarum*, although the gastropod is perhaps less sensitive than *P. promelas*. However, the same authors reported that *P. antipodarum* might be *more* sensitive to xenooestrogens than *P. promelas*. Exposures to up to 100 µg L^{-1} OP and BisA also led to concentration responses, with peak embryo production at five and 25 µg L^{-1} respectively (Jobling *et al.*, 2004). BisA induced embryo production in *P. antipodarum* at concentrations as low as 1 µg L^{-1}, compared with a LOEC of 1,280 µg L^{-1} reported for *P. promelas* (Jobling *et al.*, 2004).

BisA and OP cause similar effects in embryo production when taken up from sediments (Duft *et al.*, 2003). LOECs for *P. antipodarum* exposed to spiked sediments were 1 ng kg^{-1} for both compounds, which is in broad agreement with effective concentrations for aqueous exposure reported by Jobling *et al.* (2004), although route of uptake might differ.

Gastropods clearly have some potential as biomarkers of ED exposure. However, it might be argued that increased embryo production is not an adverse effect and further work is required to demonstrate this. Regardless, cessation of embryo at higher oestrogen/xenoestrogen concentrations is an adverse effect, although clearly not as sensitive as induction.

[1] River water that had been stripped of organic material using activated carbon

Pulmonates

In gastropods, most ED research has focussed on prosobranchs, presumably due to the TBT/imposex link. There has been some interest in ED effects in pulmonate gastropods, but so far effects seem less pronounced. Czech *et al.* (2001) exposed the freshwater pond snail *Lymnea stagnalis* to TBT, β-sitosterol (a plant oestrogen) and NP, at concentrations up to to 100 ng L^{-1} for 12 weeks. Effects only occurred at the highest exposure concentrations and varied among the contaminants: TBT caused toxic effects in the lungs, whereas β-sitosterol caused atrophy of the albumen gland (Czech *et al.*, 2001). Since β-sitosterol affected a sex gland, ED might be suspected, but there was no evidence of a mechanism to support this. More significantly, exposure to NP led to reduced egg-mass production. This finding contradicts the effect of OP in *M. cornuarietis* reported by Oehlmann *et al.* (2000); however, exposure to oestradiol (50-300 ng L^{-1}) also suppresses egg-mass production in *Planorbarius corneus* (unpublished report, Environment Agency).

Although oestrogen and xenoestrogen concentrations used in studies with pulmonates are high compared with those used in prosobranch studies, the effects reported for the latter are potentially more obviously adverse than those reported for pulmonates. For example, a 10 ng L^{-1} exposure to EE2 led to more abnormal eggs and a 1 ng L^{-1} led to more malformed embryos (and subsequent mortalities) (Casey, 2004). Although not proven, these negative effects on eggs and embryos are potentially more detrimental to snail populations than the increased egg production reported in prosobranchs.

Pulmonate gastropod species are more productive than prosobranchs and easier to culture in the laboratory. When the potential detrimental effects of EDCs on pulmonate reproduction are considered, pulmonates are potentially useful candidates for screening and monitoring purposes.

5 Other invertebrates

Studies using crustaceans, molluscs and insects dominate the scientific literature on the effects of EDCs (comprising more than 80 per cent of all published reports found in our literature search). In the Environment Agency review (1999), insects and crustaceans featured most, with less information provided on molluscs. The review also reported on echinoderms, coelenterates, porifera, acoelomates and aschelminths. Our literature search revealed that research continues on echinoderms, and also (to a lesser degree) the porifera (sponges), cnidarians (anemones and coral), ascidians, nematodes and various worm species. However, most of the studies in these invertebrate groups are laboratory bioassays, measuring impacts after exposure to EDCs. There is very little new information on the endocrinology of the animals.

5.1 Echinoderms

Current understanding of the endocrine system of echinoderms is poor, although there is general agreement that sex steroids play a role in a number of sensitive processes, including reproduction, development and regeneration (Sugni *et al.*, 2007). But although echinoderms do possess vertebrate-like steroids such as progesterone, testosterone, androstenedione, oestrone, and oestradiol, these hormones are not always present in their active forms. For example, most of the oestradiol and testosterone is usually in esterified form (Lavado *et al.*, 2006). Furthermore, levels of these steroids vary seasonally according to stage of the reproductive cycle (Sugni *et al.*, 2007)

Most ED research in echinoderms is targeted at early developmental stages, such as embryonic or larval stages, because these are considered more sensitive than adults from this point of view.

5.1.1 Sea urchins

Development of sea urchin embryos has gained support as a useful bioassay for the marine environment. In *Paracentrotus lividus,* sperm showed reduced ability to fertilise eggs following exposure to triphenyl tin (TPT), and embryos developed skeletal deformities (Moschino and Marin, 2002). Embryos died following exposure to high (10 μg L^{-1}) concentrations of TPT. Sperm were more sensitive than embryos, and so might be a useful bioassay, although this has not been demonstrated. However, Roepke *et al.* (2005) used a similar urchin embryo assay to detect effects of EDCs, in this case steroids and industrial compounds. *Strongylocentrotus purpuratus* and *Lytechinus anamesus* were exposed to 4OP, BisA, TBT and o,p-DDD, as well as oestrone (E1), oestradiol (E2), oestriol (E3), progesterone (P4) and EE2. As with Moschino and Marin (2002), development to the pluteus stage was a key indicator of effect; this is the stage 96 hours after fertilisation. TBT and 4OP were the most potent compounds, followed by E2 and EE2; E1 and E3 were much less potent (Roepke *et al.*, 2005). Again, the urchin embryo test appears to have potential, but more evidence is required.

Finally, The European Union's 'COMPRENDO' project reported EDC effects on gonad development and egg diameter in sea urchins. Gonad development results varied according to which EDC the urchins were exposed to; egg diameter was considered to be the most sensitive reproductive end-point (Sugni *et al.*, 2007).

5.1.2 Crinoids

Crinoids have the ability to regenerate limbs that have been lost due to predation or other physical pressures. The regenerative process requires a number of crucial biological/physiological processes (for example, cell proliferation, morphogenesis, differentiation, tissue renewal), and these are apparently extremely sensitive to anthropogenic pressures (Carnevali *et al.*, 2001a; Sugni *et al.*, 2007). It is unsurprising, therefore, that limb regeneration has been investigated as an effective ED bioassay endpoint. Species investigated include *Antedon mediterranea* (Carnevali *et al.*, 2001a, 2001b) and *Paracentrotus lividus* (Sugni *et al.*, 2007).

Arm regeneration is expressed as increase in length with time and varies following exposure to EDCs (triphenyltin, methyltestosterone, fenarimol, and DDE) (Lavado *et al.*, 2006). Exposure of *P. lividus* to triphenyltin, methyltestosterone, and fenarimol all caused accelerated limb growth, but DDE exposure caused no effect on limb regeneration (Lavado *et al.*, 2006). Furthermore, responses were not dose dependant. Furthermore, the study by Lavado *et al.* (2006) suggests that oestradiol is implicated in limb regeneration because in those treatments where limb regeneration was affected, there was a corresponding increase in oestradiol titres from *P. lividus.*

Polychlorinated biphenyls (PCBs) have been shown to affect regenerative arm growth in crinoids, whereby the newly grown arm is deformed. In addition, growth rate may be accelerated and there may be large scale cell proliferation and rearrangement of tissues in the stump (Carnevali *et al.*, 2001). All of these effects may be due to endocrine processes (as stated, the endocrine system is inextricably linked to growth), but this has not been proven. An embryo development test has also been developed, but only tested with TBT (Novelli *et al.*, 2002). The test essentially assessed the toxicity of TBT to crinoid sperm and embryos in terms of survival (Novelli *et al.*, 2002).

Although these studies demonstrate that limb regeneration is impacted by chemicals known to be EDCs, the exact mechanism (endocrine mediated or other) is still unclear (Sugni *et al.*, 2007)

5.2 Ascidians

Comparatively little research about ED in sea-squirts (ascidians) has been published, other than to describe the effects of TBT, which is thought to impair thyroxine metabolism and affect metamorphosis in *Ciona intestinalis* (Patricolo *et al.*, 2001).

5.3 Cnidarians

Compared with a number of the other invertebrate phlya, little work has been done to study the effects of EDCs on cnidarians (corals, jellyfish, anemones and so on). Tarrant (2007) provides a review of hormone signalling in cnidarians, but comments that they are so poorly characterized that no endpoints for an EDC bioassay have been identified. Nevertheless, some exposure/effect research is documented for cnidarians.

Steroid oestrogens (of predominantly human origin) have been shown to affect corals. Certain corals (such as *Montipora capitata*; a scleractinian) take up steroids from the water column that overlies the reef, but are able to metabolise oestrogens (such as oestradiol) and androgens (such as testosterone) (Tarrant *et al.*, 2003). Tissue levels of these hormones, therefore, may be of endogenous or exogenous origin (or a mixture of both). The same research group has since demonstrated that steroid hormones can

elicit effects consistent with ED in corals (Tarrant *et al.*, 2004). For example, when exposed to E2 for three weeks, *Montipora capitata* coral colonies released fewer egg-sperm bundles than controls (29 per cent decrease). Fragments ('nubbins') of another species, *Porites compressa*, exposed continuously to oestrone for two to eight weeks had lower (13 to 24 per cent) skeletal growth rates than controls; large coral nubbins that were treated with oestrone had thicker tissue. Biological activity of steroidal oestrogens had not been reported in coral prior to this study (Tarrant *et al.*, 2004). These findings are of particular interest, given the Environment Agency's current interest in steroid oestrogens from STWs. A number of STWs discharge their treated effluents to sea, but it is now recognised that most STW treatments do not remove these steroids completely, so it is possible that marine invertebrates inhabiting areas close to such discharges are being exposed to steroids (see Gross-Sorokin *et al.*, 2005). However, given the vast dilution of effluents discharged to sea, impacts in the natural environment are thought to be small.

Hydroids (specifically the genus *Hydra*) are the cnidarians used most widely in toxicity testing, using endpoints such as sublethal endpoints are budding, polyp structure and polyp regeneration (Tarrant, 2007). This is reflected in their prominent use in ED studies. Effects of BisA, EE2 and the drug CBZ have been investigated in hydroids (*Hydra* spp.) (Pascoe *et al.*, 2002; Quinn *et al.,* 2004). Exposure to either BisA or EE2 (at levels greater than 58 and 48 µg L^{-1}, respectively) caused malformation of the polyps in *Hydra vulgaris* (Pascoe *et al.,* 2002). However, regeneration of polyps was only affected at 460 and 150 µg L^{-1} for BisA and EE2 respectively, whilst reproduction was only affected at 500 µg EE2 L^{-1} (Pascoe *et al.*, 2002). Clearly these concentrations are not environmentally realistic, suggesting that hydroids are comparatively insensitive to these EDCs. Quinn *et al.* (2004) demonstrated that *Hydra attenuata* probably has MFO capabilities, since heme oxidase and lipoid peroxidation were induced following exposure to environmentally realistic concentrations of CBZ (Quinn *et al.*, 2004).

Although several aspects of cnidarian biology make them suitable candidates for toxicity testing, without a mechanistic understanding of cnidarian regulatory pathways, it will be extremely difficult to separate signal disruption from other stresses (Tarrant, 2007).

5.4 Sponges

Very few studies using sponges have been published in ED research (in fact, our search only found one paper). Hill *et al.* (2002) exposed two species of freshwater sponge to ethylbenzene, NP, and BisA and reported a reduction in growth rates and abnormalities in the vascular system (which is water-based in sponges). The bioassay is suggested as a useful potential tool, but a great deal more evidence is required to support this assumption.

5.5 Worms

There have been several updates on the endocrine systems of annelid worms since the 1999 report, predominantly in hormone receptor identification. Argininevasopressin (AVP)/oxytocin (OT) superfamily peptides are involved in the maintenance of water and electrolyte homeostasis in the leech *Theromyzon tessulatum,* as well as reproduction. Levoye *et al.* (2005) characterised the receptors for these peptides and showed that they are stage specific, with a weak expression after the two first blood meals, but greatly increased after the last blood meal during the period of sexual maturation; AVP/OT then disappears after egg laying. Additionally, Tanega *et al.* (2004) identified a stanniocalcin (STC) receptor in freshwater leeches. STC is generally accepted as a

vertebrate hormone, but Tanega *et al.* found STC-like proteins (and receptors) in the leeches.

In terms of bioassays, Jha and co-workers (Jha *et al.,* 2000a;2000b; Hagger *et al.*, 2002) developed an integrated suite of tests to examine genotoxicity, cytotoxicity and development in a polychaete worm (*Platynereis dumerilli*; a rag worm)[2]. The endpoints were frequency of sister chromatid exchanges and chromosomal aberrations from metaphase spreads; proliferative rate index of the growing embryo-larval cells using 5-bromodeoxyuridine labelling of the chromosomes or fluorescence plus Giemsa staining technique for geno- and cytotoxicity respectively. The authors highlight the importance of genotoxic assessment, since this was found to be most closely related to development and survival (Jha *et al.,* 2000a;2002b; 2002; Hagger *et al.*, 2002).

Another bioassay, measuring reproductive output of *Tubifex tubifex* via cocoon deposition, was developed to identify ED. For example, when exposed to sediments spiked with 4NP, *T. tubifex* deposited fewer cocoons and produced fewer offspring (Bettinetti and Provini, 2002).

5.6 Nematodes

Nematodes, predominantly the species *Caenorhabditis elegans*, are widely used in toxicity studies. Although hormonal pathways are thought to regulate physiological processes in nematodes, a definitive nematode endocrine system has not been identified (Höss and Weltje, 2007). Most work on ED in nematodes has concentrated on *C. elegans*.

Recent research on nematode endocrine systems has shown that Nematodes are able to metabolize sterols (Chitwood, 1999), that *C. elegans* has steroid/thyroid hormone receptor genes (Kostrouch *et al.*, 2005), and that free-living nematodes have oestrogen-binding proteins (Hood *et al.*, 2000). Also, Vtg is present in nematodes, and involved with the transport of cholesterol to the oocyte (Matyash *et al.* 2001).Most recently Motola *et al.* (2006) identified two 3-keto-steroids as ligands for the nuclear receptor DAF-12, which regulates dauer formation and reproduction in *C. elegans* .

Although the endocrinology of nematodes is not clearly understood, it is likely that nematodes are will be impacted by EDCs (Höss and Weltje, 2007). Consequently, nematodes have been used in a number of ecotoxicological and biomarker studies to determine the effects of EDCs, including steroids and industrial-type chemicals. For example, *C. elegans* exposed to E2, testosterone and DES had reduced fecundity (if cultured in the absence of cholesterol) (Tominaga *et al.*, 2003). Perhaps more importantly, testosterone accumulated over generations (Tominaga *et al.*, 2003). Furthermore, testosterone exposure was shown to cause increased vitellogenin synthesis in *C. elegans* (Custodia *et al.*, 2001). Indeed, Custodia *et al.* (2001) used a DNA microarray analysis to investigate the effects of testosterone and progesterone on the expression of vitellogenin, cytochrome P450 and glutathione s-transferase (GST) genes, and suggested that *C. elegans* might prove to be a suitable biomarker of ED. Although Ura *et al.*, (2002) exposed *C. elegans* to a number of substances purported to be EDCs (such as NP, BisA, BaP), the substances were treated as standard toxicants and the authors only recorded lethal effects.

Other endpoints used in EDC exposure experiments with nematodes include growth, reproduction, fecundity, number of germ cells, vitellogenin expression and oestrogen binding; for a detailed list of effects of refer to Höss and Weltje (2007) and references

[2] The same assay is also suitable for mussels.

therein. However, as with other Phyla, not all of these responses are necessarily caused specifically by disruption of the endocrine system.

6 Environmental evidence

Substantial evidence now shows that a number of chemical substances, from a variety of sources, exert ED effects in invertebrates. Some are natural substances (such as steroid hormones excreted by humans), some are industrial chemicals that have ED properties by chance (such as NP) and others are chemicals deliberately produced and released into the environment for their ED properties (such as juvenile hormone analogue insecticides). However, while there is considerable evidence from laboratory toxicity studies that these substances have the ability to cause effects consistent with ED in invertebrates, and some evidence that ED is occurring in individuals in the natural environment, evidence of population level effects of ED in the natural environment is apparently scarce. This was also highlighted in the Environment Agency 1999 report.

TBT-induced imposex in female dogwhelks (*Nucella lapillus*) is arguably the best-known example of ED in natural populations. TBT was the base compound for antifouling paints, but female dogwhelks in the vicinity of treated boats and marinas were found to have non-functional male sexual characteristics growing on top of the normal female structures, rendering many of them incapable of reproduction. There are a great many published examples of imposex due to TBT exposure; it was well documented in the 1999 Environment Agency review and many publications have reported this phenomenon since the review. Given that the aim of this report is to document recent advances in ED research, this topic is not dealt with further here.

One of the main sources of concern for EDCs in the UK is sewage treatment work effluents. These contain human steroid hormones; however, they also contain a number of other xenobiotics, some of which may exert ED effects. Risk assessment of the ED effects of mixtures is being addressed in another *Horizon scanning* report and so mixed effluent evidence will only be covered in minor detail here. However, treatment of effluent mixtures highlights the problem of attributing cause and effect relationships to potential EDCs. For example, Zulkosky *et al.* (2002) reported that reproductive output of the marine amphipod *Leptocheirus plumulosus* was reduced by 50 per cent when exposed to sewage-impacted sediment. However, although nonylphenol ethoxylate (NPEO) concentrations in the sediment were as high as 44.2 $\mu g\ L^{-1}$, laboratory exposures to NP (in isolation) did not affect reproduction. In addition to natural causes, reduced reproductive output can be caused by a number of xenobiotic substances and is not necessarily the result of ED. In such cases, definite identification of the cause of reduced reproductive output is required to confidently conclude that ED is occurring.

Another example of amphipods affected by effluents known to contain EDCs is the study of Gross *et al.* (2001). Female *Gammarus pulex* from the River Lea in England (which had already been shown to cause oestrogenic responses in fish downstream of STWs from the presence of E1 and E2 in the effluent) had malformed oocytes and grew significantly shorter than unaffected females. No malformation of male gonadal structure was seen, which is somewhat surprising since often it is the males that develop female characteristics following exposure to oestrogenic substances. However, effluent exposed males grew shorter than unimpacted males, to such an extent that instead of being up to 30 per cent larger than the females (as in unaffected populations), they were the same size as females. This result suggests that exposure to effluents from STWs might reduce the reproductive success of *G. pulex,* since the larger size of the male is advantageous in establishing and maintaining precopular pairing with the female. This is supported by the fact that fewer precopular pairs were found on sites below the STWs (Gross *et al.*, 2001). Whether the reduced number of

successfully paired amphipods affected total recruitment or had long-term implications for survival of the population is not known.

STW effluent has also been reported to impact on molluscs. For example, Blaise *et al.* (1999) reported that at a sampling station downstream of a sewage treatment works outfall and a marina (Baie Eternite, Saint-Lawence Estuary, Canada), clams were experiencing a delay in the onset of gametogenesis. The authors hypothesised that the energy status of the clams was being affected, and this in turn was affecting vitellogenesis. Glycogen levels in the gonads of affected clams were significantly higher than controls (Blaise *et al.*, 1999), indicating dysfunction in gonadal consumption and consequent vitellogenesis (Gauthier-Clerc *et al.*, 2002).

Gagne *et al.* (2001a) looked for specific endocrine effects in freshwater mussels (*Elliptio complanata*) downstream of STW outfalls. It was hypothesised that the mussels were being affected by coprostanol (a steroid found in human faeces), which is bacterially converted from the high levels of cholesterol found in sewage effluents (Gagne *et al.*, 2001a). They found that the mussels caged downstream had much higher levels of coprostanol than those left upstream for 62 days. This is to be expected, because mussels filter feed particulates in the water and as such are prone to bioaccumulation of lipophillic compounds like coprostanol.

Aside from these few examples, no other new examples of ED occurring in wild populations were found in the literature search. A large majority of ED studies in invertebrates are conducted in the laboratory, following the deliberate exposure of the organism in question to an EDC. Furthermore, none of the examples presented here that describe ED occurring in natural populations demonstrate lasting deleterious impacts on natural populations. Individuals are impacted by EDCs, but impacts at the population or ecosystem level have not been demonstrated.

Ecological/mathematical modelling may provide information on the likely effects on populations (by estimating impacts on survivorship, fecundity, reproductive output and so on), and is a research area that is expanding. For example, Kuhn *et al.* (2001) developed a matrix model to extrapolate the results from a chronic laboratory exposure experiment to population-level responses in mysids. The widely-used *Americamysis bahia* was exposed continuously to para-NP over three generations and the effects compared with those predicted by the model. The model proved to be very accurate, predicting a population response at 16 µg L^{-1} compared with the three generation test value of 19 µg L^{-1} (Kuhn *et al.*, 2001).

Another method to estimate 'real world' scenarios with EDCs is to use mesocosm-type experiments. These use natural samples of water or sediment as the exposure medium. For example, Sundelin *et al.* (2000) assessed maturation of the amphipod *Monoporeia affinis* following exposure to sediments collected from control and two contaminant-impacted sites (one site received run-off from a refuse dump with unidentified contaminants; the other was from an urban site containing heavy metals, PAHs and PCBs). Amphipods exposed to sediments that were contaminated with refuse effluent had disrupted reproductive output: 50 per cent of females had delayed sexual development, of which 28 per cent were immature. Fecundity and fertilisation success were also reduced in these amphipods. Males were similarly affected, showing delayed sexual maturation and reduced secondary sexual characteristics (Sundelin *et al.*, 2000). Mesocosm experiments such as these are useful as they use real sediments (or water) from natural sites and, assuming that the organisms do not avoid the impacted site (to date there is no evidence that organisms can detect EDCs in the environment), demonstrate likely effects on natural populations. Population effects *per se* (such as population decline or extinction), however, still need to be modelled.

With regard to the regulatory monitoring of EDCs, one of the more difficult problems to overcome is that mixed-gender or intersex individuals can occur in a great many

invertebrate species without necessarily being exposed to EDCs. Whilst this is also true of fish species, the occurrence is more widespread in invertebrates. For example, imposex has been reported in both male and female Japanese freshwater crabs *Geothelphuma daani* inhabiting uncontaminated mountain streams in Japan (Takahashi *et al.*, 2000). Similar findings were reported for *Gammarus fossarum* by Jungmann *et al.* (2004). Intersex individuals were found in several *G. fossarum* populations; however, not all of the affected populations inhabited contaminated sites. Although the cause of intersex in the uncontaminated site was not identified, transplant experiments indicated that intersex was caused by something in the exposure water (Jungmann *et al.*, 2004).

In addition to intersex occurring naturally within invertebrate populations, they may also occur in some invertebrate species due to infection by parasites. For example, in the estuarine amphipod *Gammarus duebeni*, males infected with the microsporidain parasite *Nosema granulosis* are converted into females (Rodgers-Gray *et al.,* 2004). Males develop fully functioning ovaries as a result of infection by the parasite, which is thought to manipulate the host's sex by inhibiting differentiation of the androgenic gland (Rodgers-Gray *et al.,* 2004). This leads to prevention of androgenic gland hormone production, the lack of which prevents the amphipod differentiating into a male. Although infection causes fully functioning ovaries to develop, complete feminisation does not occur. Almost 90 per cent of intersex individuals within the population studied by Rodgers-Gray *et al.* (2004) were infected by *N. granulosis,* suggesting that this parasite is the main reason for intersex in *G. duebeni*.

A lack of evidence of ED occurring in the natural environment was highlighted in the Environment Agency (1999) review. There have been very few reports of ED occurring in natural invertebrate populations since the 1999 report. While laboratory exposures and mesocosm experiments clearly identify the potential for ED in natural populations, evidence of ED actually occurring remains limited to a few isolated cases. Since our understanding of EDCs and identification of the sources are improving, records of ED occurring in the natural environment are likely to increase. For example, studies in fish have demonstrated that ED occurs following exposure to the steroid hormones discharged in STW effluent (see Gross-Sorokin *et al.*, 2005). Assessment of invertebrate populations downstream of STWs is likely to demonstrate that ED occurs in the invertebrate populations as well as fish (see Gross *et al.*, 2001). However, although monitoring for affected invertebrates in receiving waters downstream of STW effluents was highlighted as a regulatory need in the 1999 review, there has been little progress in this area. Identification of ED occurring in the natural environment, and in particular impacting at the population level, remains a research need.

7 Discussion and conclusions

7.1 Have the recommendations from Environment Agency (1999) been implemented?

One of the aims of this report is to determine whether any of the recommendations from the Environment Agency 1999 review have been followed up, and whether any of the knowledge gaps identified have been filled. The following section quotes the recommendation from the Environment Agency (1999), and then assesses it in terms of current knowledge (numbers refer to the recommendation number as given in the executive summary of Environment Agency, 1999) (see Appendix 1).

30. *The detection of effects of exposure to contaminants in both natural and laboratory populations of invertebrates, and the assessment of the ability of chemicals to interfere with endocrine-dependent processes (growth, reproduction, behaviour), does not require a detailed understanding of the endocrinology of the organism concerned.*

This statement remains valid, and this is reflected in the research activity from the last seven to eight years. Although understanding of the endocrine systems of invertebrates has increased, the wide variety of systems means we are still a long way from having a complete or detailed understanding of the endocrine systems of all invertebrates. However, the research area of ED is extremely active and a great many publications report the effects of EDCs or ED in organisms for which we still lack detailed knowledge of their endocrine system. This means, however, that some chemicals may be attributed to interfering with the endocrine system of an organism when, in fact, it elicits its effects via another means. This is likely to remain the case until detailed knowledge is available on the endocrine system of all invertebrates (or at least for a range of invertebrates considered to be representative of all invertebrates).

31. *However, an understanding of the endocrinology of relevant organisms is important if the mechanisms by which environmental contaminants elicit effects are to be accurately attributed. Supporting work on mechanisms is therefore important to develop definitive evidence for which chemicals are invertebrate endocrine disruptors.*

Again, this statement is true, and the recommendation that research on mechanisms is needed has partially been fulfilled. Considerable research effort is being made into understanding the endocrine system and molecular biology of certain invertebrate groups/species. For example, the fruit-fly *Drosophila melanogaster* has received much research attention because of its widespread use in genetic studies. Economically important species such as mosquitoes (for the development of insecticides) have also been the focus of many studies; an understanding of the endocrine systems of molluscs has also developed. However, less effort is being made on the study of basic endocrinology and mechanisms by which EDCs elicit their effects in crustaceans (although certain groups, such as mysids, are being investigated), echinoderms and other aquatic phyla. Further supporting work is required even for the better studied phyla, and increased effort to gather supporting evidence is required for the less well-studied phyla.

32. *The major research need in the first instance is for field surveys to establish whether there is any evidence for endocrine disruption in individuals or populations. Systematic biological monitoring is needed in situations where chemicals with endocrine disrupting capability are most likely to be found, notably in rivers downstream of sewage or industrial effluents and in the neighbourhood of sewage outfalls in the marine environment.*

In general, this recommendation remains unfulfilled. While there have been several reported incidences of ED occurring in natural invertebrate populations, there has not been a targeted research effort to identify ED in the natural environment. With the exception of the studies of Gross (Gross-Sorokin *et al*., 2001) and co-workers, there have been no systematic studies of impacts on invertebrate populations downstream of STWs. Some studies have reported mesocosm-type studies of effects of sewage sludge, but to date there remains limited information on ED in natural populations; what evidence we do have seems to be only for amphipods.

33. *Current biological water quality monitoring should then be extended to take into account appropriate indicators of endocrine disruption.*

Although EDCs are now a high profile group of chemicals, recognised to affect water quality, current monitoring practices still have no reliable means of testing for EDCs in the natural environment. This remains a key requirement for the successful management of the aquatic environment. Several European directives (such as the Water Framework Directive and the Habitats and Birds Directive) demand improved water quality in our rivers, lakes and seas. EDCs are among the main chemical groups of concern. so it is important that regulators have means to test and monitor the aquatic environment for these substances. The Environment Agency is currently running a number of projects that will help to achieve this.

34. *To do this effectively, it will be necessary to identify a range of "sentinel" organisms together with appropriate endpoints/indicators of endocrine-disrupting activity.*

A number of key invertebrate groups, through their previous good history as test organisms, are likely candidates for use as 'sentinel' organisms. However, at present there is no defined suite of biomarker organisms that can be used to fulfil this recommendation. Nor is there an accepted list of endpoints.

35. *In the freshwater environment, sentinels, and subjects chosen for bioassay, should include representatives of the Annelida, Mollusca, Crustacea, and Insecta and for the marine environment Coelenterata, Annelida, Mollusca, Crustacea and Echinodermata should be represented.*

The phyla suggested here remain sensible, but as stated above, accepted representatives of these phyla are currently lacking. For the freshwater environment, certain species are good candidates. For example, *Daphnia* and *Gammarus sp.* are strong candidates for the Crustacea; chironomid and mosquitoes are good candidate for insects. Mysids and copepods are strong candidates for marine crustaceans. Representatives from other phlyla are less obvious. However, a range of candidates is required to ensure that all environments are represented; and it is preferable if bioassay organisms are representative of the natural invertebrate assemblage. Such a range of sentinel and bioassay organisms has still not been identified. One current research project being run by the Environment Agency is to assess the suitability of various mollusc species as testing organisms; the results will help in the creation of such a suite of sentinel/bioassay organisms.

38. *What must be determined is whether:*

a: it is appropriate to instigate a test regime (or continue with existing test strategies) that identifies effects without necessarily identifying the route by which those effects occur, or

b: whether it is important to discriminate between compounds which act via the endocrine system and those which do not.

This question remains unanswered, and current thinking seems to support both suggestions. Without detailed knowledge of all invertebrate endocrine systems, it is not possible at present to identify the route/mechanism of effect. At present, it seems acceptable to attribute the effects of compounds of recognised ED potency to endocrine disruption, without identifying the precise pathway or mechanism. Obviously it would be preferable to identify the precise mechanism, but if such information is lacking, yet there is clear evidence that an impact is occurring, management action should be taken.

In terms of ecological relevance, tests that examine reproduction and other life-cycle criteria are clearly important, regardless of whether we actually know the endocrine mechanism by which they are disrupted. Biochemical tests such as mixed-function oxidase and vitellin induction are useful as screening tests for ED, but the ecological relevance of positive results has not yet been proven. This is an important point, as it contradicts the basis of risk assessments currently being developed for fish by the Environment Agency. In fish, vitellogenin induction is not accepted in isolation as an endpoint indicating remediation requirements; instead, reproduction and population effects of EDCs are currently being sought. In invertebrates, however, although life-cycle tests are considered important (for example, the US EPA's two-generation life-cycle test,) there is still a large resource investigating biochemical endpoints.

Further to this question, Zou (2005) introduced the term invisible endocrine disruption to describe how EDC is likely to be happening, even though there is not sufficient understanding of the relevant endocrinology to prove ED definitely. Zou (2005) supplemented his argument by reviewing different ways how this disruption might happen, e.g. by affecting synthesis and release of hormones, metabolism, or binding to receptors.

The importance of understanding the mechanisms of ED in invertebrates is also being considered by the OECD (Gourmelon and Ahtiainen, 2007).

39. *The former strategy involves the minimum of investment in new techniques and test methods; the latter strategy would require considerable investment in research to identify indicators of disruption in specific elements of the endocrine systems of a diverse range of invertebrate species.*

Given that established tests require little further investment (financial or time), and often have the approval of regulatory agencies or international scientific committees, it is not surprising that these remain the most frequently used; there are comparatively few new techniques published compared with studies using existing methods. Some research areas are promising in terms of new techniques, notably molecular biology and genomics, and these continue to expand.

40. *The most appropriate use of research resources might encompass a dual track approach - continued testing with existing protocols which include response measures likely to detect effects of endocrine disruption in addition to other modes of toxicity, together with a research programme targeted at identifying the mechanisms underlying effects attributable to compounds suspected of endocrine disrupting capabilities.*

This final statement from the Environment Agency (1999) document appears to be the way that ED research has progressed since 1999. Protocols in use in 1999

remain in widespread use today, although some may have undergone minor revisions and improvements. There has been continued interest in gaining a more detailed understanding of invertebrate endocrine systems, their mechanisms, and the modes of action of EDCs. This is likely to remain the case. Regulators are under pressure to demonstrate, monitor and legislate against the pressures of anthropogenic chemicals, so existing tools and techniques are required to be able to do this. Notwithstanding, there is ongoing research into new tools and techniques that will help identify the impacts of EDCs.

7.2 Recommendations and future direction of ED research in invertebrates

The Organisation for Economic Co-operation and Development (OECD) Test Guidelines Programme was established to co-ordinate international harmonisation and validation of test methods to evaluate effects of chemicals. EDCs are included in the chemicals for which tests have been developed and validated. Invertebrate reproduction tests that are undergoing validation by the OECD include copepod, mysid and daphnid tests (Gourmelon and Ahtiainen, 2007). Other potential tests to be developed include test for chironomids (Taenzler *et al.*, 2006) and prosobranch snails (Duft *et al.*, 2006). Issues that the OECD are currently addressing are:

- what is the importance of mechanistic information in regulating chemicals?, and;
- what is the best way to address the issue of possible endocrine disruption in invertebrates while integrating these tests in a regulatory scheme? (Gourmelon and Ahtiainen, 2007).

Additionally, in the recent special issue of the journal Ecotoxicology, Hutchinson (2007) made four recommendations for ED research in aquatic invertebrates. These were:

- increased use of invertebrate populations in the natural environment to study potential impacts of anthropogenic substances;
- the prioritisation of selected species of invertebrates for testing in the laboratory;
- the validation of biomarkers that identify specific ED mechanisms; and
- an updated list of reference EDCs for use in ED toxicity testing that affect invertebrates via a variety of mechanisms.

In summary, it is clear that a great deal of time and financial resources have been targeted at investigating ED in invertebrates. Progress made has been variable, depending on the invertebrate Phylas being investigated. In terms of using invertebrates for regulatory purposes, while consistent progress is being made on testing methods [for example, the OECD guidelines (OECD, 1998; 2004a; 2004b; 2006)], there has been less progress on the development of reliable biomarkers for the natural environment. The requirement to legislate and regulate EDCs remains important (see Environment Agency, 2007b) and so it is likely that laboratory studies of EDC effects will continue in parallel with research to better understand invertebrate endocrinology. Many of the recommendations from Environment Agency (1999) still have considerable merit for ED research in invertebrates and, if taken forward with those of the OECD and those made by Hutchinson (2007), the potential of invertebrates for use in EDC regulation and monitoring should be realised.

References

ADAM, G., PERRIMON, N. AND NOSELLI, S., 2003. The retinoic-like juvenile hormone controls the looping of left-right asymmetric organs in Drosophila. *Development*, 130 (11), 2397-2406.

ANDERSEN, H.R., WOLLENBERGER, L., HALLING-SORENSEN, B. AND KUSK, K.O., 2001. Development of copepod nauplii to copepodites - A parameter for chronic toxicity including endocrine disruption. *Environmental Toxicology and Chemistry*, 20 (12), 2821-2829.

ATIENZAR, F.A., BILLINGHURST, Z. AND DEPLEDGE, M.H., 2002. 4-n-nonylphenol and 17-beta estradiol may induce common DNA effects in developing barnacle larvae. *Environmental Pollution*, 120 (3), 735-738.

BALDWIN, W.S., BAILEY, R., LONG, K.E. AND KLAINE, S., 2001. Incomplete ecdysis is an indicator of ecdysteroid exposure in Daphnia magna. *Environmental Toxicology and Chemistry*, 20 (7), 1564-1569.

BARATA, C., PORTE, C. AND BAIRD, D.J., 2004. Experimental designs to assess endocrine disrupting effects in invertebrates - A review. *Ecotoxicology*, 13 (6), 511-517.

BARTLETT, A.J., BORGMANN, U., DIXON, D.G., BATCHELOR, S.P. AND MAGUIRE, R.J., 2004. Accumulation of tributyl tin in *Hyalella azteca* as an indicator of chronic toxicity: Survival, growth, and reproduction. *Environmental Toxicology and Chemistry*, 23 (12), 2878-2888.

BECKAGE, N.E., MARION, K.M., WALTON, W.E., WIRTH, M.C. AND TAN, F.E., 2004. Comparative larvicidal toxicities of three ecdysone agonists on the mosquitoes *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles gambiae*. *Archives of Insect Biochemistry and Physiology*, 57 (3), 111-122.

BETTINETTI, R. AND PROVINI, A., 2002. Toxicity of 4-nonylphenol to *Tubifex tubifex* and *Chironomus riparius* in 28-day whole-sediment tests. *Ecotoxicology and Environmental Safety,* 53 (1), 113-121.

BILLINGHURST, Z., CLARE, A.S., MATSUMA, K. AND DEPLEDGE, M.H., 2000. Induction of cypris major protein in barnacle larvae by exposure to 4-n-nonylphenol and 17 beta-oestradiol. *Aquatic Toxicology*, 47 (3-4), 203-212.

BILLINGHURST, Z., CLARE, A.S., AND DEPLEDGE, M.H., 2001. Effects of 4-n-nonylphenol and 17-beta estradiol on early development of the barnacle Elminius modestus. *Journal of Experimental Marine Biology and Ecology*, 257 (2), 255-268.

BIRCHENOUGH, A.C., BARNES, N., EVANS, S.M., HINZ, H., KRONKE, I. AND MOSS, C., 2002. A review and assessment of tributyltin contamination in the North Sea, based on surveys of butyltin tissue burdens and imposex/intersex in four species of neogastropods. *Marine Pollution Bulletin*, 44 : 534-543 JUN 2002

BLAISE, C., GAGNE, F., PELLERIN, J. AND HANSEN, P., 1999. Determination of vitellogenin-like properties in *Mya arenaria* hemolymph (Saguenay Ford, Canada): A potential biomarker for endocrine disruption. *Environmental Toxicology*, 14, 455-465.

BOUDJELIDA, H., BOUAZIZ, A., SOIN, T., SMAGGHE, G., AND SOLTANI, N., 2005. Effect of ecdysone agonist halofenozide against *Culex pipiens*. *Pesticide Biochemistry Physiology*, 83, 115–123. Cited in Soin and Smagghe (2007).

BREITHOLTZ, M. AND BENGTSSON, B.E., 2001. Oestrogens have no hormonal effect on the development and reproduction of the harpacticoid copepod *Nitocra spinipes*. *Marine Pollution Bulletin*, 42 (10), 879-886.

BREITHOLTZ, M., WOLLENBERGER, L. AND DINAN, L., 2003. Effects of four synthetic musks on the life cycle of the harpacticoid copepod *Nitocra spinipes*. *Aquatic Toxicology*, 63 (2), 103-118.

BRYAN, G., GIBBS, P., HUMMERSTONE, L. AND BURT, G., 1986. The decline of the gastropod *Nucella lapillus* around South-West England: Evidence for the effect of tributyl tin from antifouling paints. *Journal of the Marine Biological Association*, 66, 611-640.

BRYAN, G., GIBBS, P., BURT, G. AND HUMMERSTONE, L., 1987. The effects of tributyl tin (TBT) accumulation on adult dogwhelks *Nucella lapillus*: Long-term field and laboratory experiments. *Journal of the Marine Biological Association*, 67, 525-544.

CARNEVALI, M.D.C., BONASORO, F., PATRUNO, M., THORNDYKE, M.C. AND GALASSI, S., 2001a. PCB exposure and regeneration in crinoids (Echinodermata). *Marine Ecology-Progress Series*, 215, 155-167.

CARNEVALI, M.D.C., GALASSI, S., BONASORO, F., PATRUNO, M. AND THORNDYKE, M.C., 2001b. Regenerative response and endocrine disrupters in crinoid echinoderms: Arm regeneration in *Antedon mediterranea* after experimental exposure to polychlorinated biphenyls. *Journal of Experimental Biology*, 204 (5), 835-842.

CASEY, D., 2004. *The effect of the synthetic oestrogen ethinyl-estradiol on the pond snail Lymnaea Stagnalis*. Unpublished Ph.D. Thesis. University of Cardiff.

CHEN, S.H., LIN, C.Y., AND KUO, C.M., 2005. In silico analysis of crustacean hyperglycemic hormone family. *Marine Biotechnology*, 7, 193–206. Cited in LeBlanc (2007).

CHENG, C. AND LIU, L., 2004. Gametogenesis in the imposex-affected oyster drills, *Thais clavigera* and *Thais rufotincta*. *Journal of the Fisheries Society of Taiwan,* 31, 55-65.

CHITWOOD, D.J., 1999. Biochemistry and function of nematode steroids. *Critical Reviews in Biochemistry and Molecular Biology*, 34, 273–284. Cited in Höss and Weltje (2007).

CLARKE, A.C., DEL CAMPO, M.L. AND EWER, J., 2004. Neuroendocrine control of larval ecdysis behaviour in *Drosophila*: Complex regulation by partially redundant neuropeptides. *Journal of Neuroscience*, 24 (17), 4283-4292.

CRIPE, G.M., MCKENNEY, C.L., HOGLUND, M.D., HARRIS, P.S., 2003. Effects of fenoxycarb exposure on complete larval development of the xanthid crab, *Rhithropanopeus harrisii*. *Environmental Pollution*, 125, 295–299.

CSTEE, 1999. *CSTEE opinion on human and wildlife health effects of endocrine disrupting chemicals, with emphasis on wildlife and on ecotoxicology test methods*. Report of the Working Group on Endocrine Disrupters of the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) of DG XXIV, Consumer Policy and Consumer Health Protection.

CUSTODIA, N., WON, S.J., NOVILLO, A., WIELAND, M., LI, C. AND CALLARD, I.P., 2001. *Caenorhabditis elegans* as an environmental monitor using DNA microarray analysis. *Environmental Hormones: The Scientific Basis of Endocrine Disruption*, 948, 32-42.

CZECH, P., WEBER, K. AND DIETRICH, D., 2001. Effects of endocrine modulating substances on reproduction in the hermaphroditic snail *Lymnaea stagnalis* L. *Aquatic Toxicology*, 53, 103-114.

DAVISON, G.P., RESTREPO, R., MARTINEZ, G., COLL, F. AND LEON, O.S., 2003. Effects of a brassinosteroid analogue to mosquito larvae. *Ecotoxicology and Environmental Safety*, 56 (3), 419-424.

DEFUR, P.L., 2004. Use and role of invertebrate models in endocrine disruptor research and testing. *Ilar Journal*, 45 (4), 484-493.

DEFUR, P.L., CRANE, M., INGERSOLL, C. AND TATTERSFIELD, L., 1999. *Endocrine disruption in invertebrates: Endocrinology, testing and assessment (Proceedings of Workshop, 1998)*. Pensacola, FL, USA: SETAC press.

DEPLEDGE, M.H. AND BILLINGHURST, Z., 1999. Ecological relevance of endocrine disruption in marine invertebrates. *Marine Pollution Bulletin,* 39, 32–38.

DODSON, S.I., MERRITT, C.M., TORRENTERA, L., WINTER, K.M., TORNEHL, C.K. AND GIRVIN, K., 1999. Dieldrin reduces male production and sex ratio in *Daphnia galeata mendotae*. *Toxicology and Industrial Health*, 15 (1-2), 192-199.

DUFT, M., SCHMITT, C., BACHMANN, J., BRANDELIK, C., SCHULTE-OEHLMANN, U., OEHLMANN, J., 2006. Prosobranch snails as test organisms for the assessment of endocrine active chemicals—an overview and a guideline proposal for a reproduction test with the freshwater mudsnail *Potamopyrgus antipodarum*. *Ecotoxicology*, 16, 169-182.

DUFT, M., SCHULTE-OEHLMANN, U., WELTJE, L., TILLMANN M., AND OEHLMANN, J., 2003. Stimulated embryo production as a parameter of estrogenic exposure via sediments in the freshwater mudsnail *Potamopyrgus antipodarum. Aquatic Toxicology*, 64, 437-449.

ELEKONICH, M.M. AND HORODYSKI, F.M., 2003. Insect allatotropins belong to a family of structurally-related myoactive peptides present in several invertebrate phyla. *Peptides*, 24 (10), 1623-1632.

ENVIRONMENT AGENCY, 1999. *Endocrine function in aquatic invertebrates and evidence for disruption by environmental pollutants*. Environment Agency: Bristol, UK.

ENVIRONMENT AGENCY, 2007a. Endocrine disruption: molecular and genomic contributions. Science Report SC030276/SR2. Environment Agency: Bristol, UK.

ENVIRONMENT AGENCY, 2007b. *Endocrine disruption horizon scanning: Current status of endocrine disruptor research and policy*. Science Report SC030276/SR4. Environment Agency: Bristol, UK.

FERNANDEZ, M.A., WAGENER, A.D.R., LIMAVERDE, A.M., SCOFIELD, A.L., PINHEIRO, F.M. AND RODRIGUES, E., 2005. Imposex and surface sediment speciation: A combined approach to evaluate organotin contamination in Guanabara Bay, Rio de Janeiro, Brazil. Marine Environmental Research, 59, 435-452.

FISHER, T., CRANE, M. AND CALLAGHAN, A., 2003. Induction of cytochrome P-450 activity in individual *Chironomus riparius* Meigen larvae exposed to xenobiotics *Ecotoxicology and Environmental Safety*, 54 (1), 1-6.

FORD, A.T., FERNANDES, T.F., RIDER, S.A., READ, P.A., ROBINSON, C.D. AND DAVIES, I.M., 2004a. Endocrine disruption in a marine amphipod? Field observations of intersexuality and de-masculinisation. *Marine Environmental Research*, 58 (2-5), 169-173.

FORD, A.T., FERNANDES, T.F., READ, P.A., ROBINSON, C.D. AND DAVIES, I.M., 2004b. The costs of intersexuality: a crustacean perspective. *Marine Biology*, 145 (5), 951-957

FORGET-LERAY, J., LANDRIAU, I., MINIER, C. AND LEBOULENGER, F. 2005. Impact of endocrine toxicants on survival, development, and reproduction of the estuarine copepod *Eurytemora affinis* (Poppe). *Ecotoxicology and Environmental Safety*, 60 (3), 288-294.

GAGNE, F., BLAISE, C., LACHANCE, B., SUNAHARA, G. AND SABIK, H., 2001a. Evidence of coprostanol estrogenicity to the freshwater mussel *Elliptio complanata*. *Environmental Pollution*, 115, 97-106.

GAGNE, F., BLAISE, C., SALAZAR, M., SALAZAR, S. AND HANSEN, P., 2001b. Evaluation of estrogenic effects of municipal effluents to the freshwater mussel *Elliptio complanata*. *Comparative Biochemistry and Physiology* C, 128, 213-225.

GAGNE, F., BLAISE, C. AND HELLOU, J., 2004. Endocrine disruption and health effects of caged mussels, *Elliptio complanata*, placed downstream from a primary-treated municipal effluent plume for one year. *Comparative Biochemistry and Physiology C*, 138, 33-44.

GAUTHIER-CLERC, S., PELLERIN, J., BLAISE, C. AND GAGNE, F., 2002. Delayed gametogenesis of *Mya arenaria* in the Saguenay fjord (Canada): a consequence of endocrine disruptors? *Comparative Biochemistry and Physiology* C, 131, 457-467.

GHEKIERE, A., VERSLYCKE, T., DE SMET, L., VAN BEEUMEN, J. AND JANSSEN, C.R., 2004. Purification and characterization of vitellin from the estuarine mysid *Neomysis integer* (Crustacea; Mysidacea). *Comparative Biochemistry and Physiology A (Molecular & Integrative Physiology)*, 138 (4), 427-433.

GHEKIERE, A., FOCKEDEY, N., VERSLYCKE, T., VINCX, M. AND JANSSEN, C.R., 2007. Marsupial development in the mysid *Neomysis integer* (Crustacea: Mysidacea) to evaluate the effects of endocrine disrupting chemicals. *Ecotoxicology and Environmental Safety*, 66, 9-15.

GOURMELON, A. AND AHTIAINEN, J., 2007. Developing Test Guidelines on invertebrate development and reproduction for the assessment of chemicals, including potential endocrine active substances—The OECD perspective. *Ecotoxicology*, 16, 161–167.

GROSS, M.Y., MAYCOCK, D.S., THORNDYKE, M.C., MORRITT, D. AND CRANE, M., 2001. Abnormalities in sexual development of the amphipod *Gammarus pulex* (L.) found below sewage treatment works. *Environmental Toxicology and Chemistry*, 20 (8), 1792-1797.

GROSS-SOROKIN, M.Y., ROAST, S.D. AND BRIGHTY, G.C., 2006. Assessment of feminization of male fish in English rivers by the Environment Agency of England and Wales. *Environmental Health Perspectives*, 114(suppl 1), 147-151.

HAGGER, J.A., FISHER, A.S., HILL, S.J., DEPLEDGE, M.H. AND JHA, A.N., 2002. Genotoxic, cytotoxic and ontogenetic effects of tri-n-butyl tin on the marine worm, *Platynereis dumerilii* (Polychaeta : Nereidae). *Aquatic Toxicology*, 57 (4), 243-255.

HAHN, T. AND SCHULZ, R., 2002. Ecdysteroid synthesis and marginal disc development in the midge *Chironomus riparius* as biomarkers for endocrine effects of tributyl tin. *Environmental Toxicology and Chemistry*, 21 (5), 1052-1057.

HAHN, T., SCHENK, K. AND SCHULZ, R., 2002. Environmental chemicals with known endocrine potential affect yolk protein content in the aquatic insect *Chironomus riparius*. *Environmental Pollution*, 120 (3), 525-528.

HILL, M., STABILE, C., STEFFEN, L.K. AND HILL, A., 2002. Toxic effects of endocrine disrupters on freshwater sponges: common developmental abnormalities. *Environmental Pollution*, 117 (2), 295-300.

HOOD, T.E., CALABRESE, E.J. AND ZUCKERMAN, B.M., 2000. Detection of an estrogen receptor in two nematode species and inhibition of binding and development by environmental chemicals. *Ecotoxicology and Environmental Safety*, 47, 74–81.

HOPKINS, P.M., 2001. Limb regeneration in the fiddler crab, *Uca pugilator*: Hormonal and growth factor control. *American Zoologist*, 41 (3), 389-398.

HÖSS, S. AND WELTJE, L., 2007. Endocrine disruption in nematodes: effects and mechanisms. *Ecotoxicology*, 16, 15-28.

HUANG, D.J. AND CHEN, H.C., 2004. Effects of chlordane and lindane on testosterone and vitellogenin levels in green neon shrimp (*Neocaridina denticulata*). *International Journal of Toxicology*, 23 (2), 91-95.

HUANG, D.J., WANG, S.Y. AND CHEN, H.C., 2004. Effects of the endocrine disrupter chemicals chlordane and lindane on the male green neon shrimp (*Neocaridina d*enticulata). *Chemosphere,* 57 (11), 1621-1627.

HUTCHINSON, T.H., 2002. Reproductive and developmental effects of endocrine disrupters in invertebrates: in vitro and in vivo approaches. *Toxicology Letters*, 131 (1-2), 75-81.

HUTCHINSON, T.H., 2007. Small is useful in endocrine disrupter assessment—four key recommendations for aquatic invertebrate research. Ecotoxicology, 16, 231-238.

JANER, G., LAVADO, R., THIBAUT, R. AND PORTE, C., 2004. Effects of 17b-estradiol exposure in the mussel *Mytilus galloprovincialis*. *Marine Environmental Research*, 58, 443-446.

JHA, A.N., HAGGER, J.A., HILL, S.J. AND DEPLEDGE, M.H., 2000a. Genotoxic, cytotoxic and developmental effects of tributyl tin oxide (TBTO): an integrated approach to the evaluation of the relative sensitivities of two marine species. *Marine Environmental Research*, 50 (1-5), 565-573.

JHA, A.N., HAGGER, J.A. AND HILL, S.J., 2000b. Tributyl tin induces cytogenetic damage in the early life stages of the marine mussel, *Mytilus edulis*. *Environmental and Molecular Mutagenesis*, 35 (4), 343-350.

JOBLING, J., CASEY, D., RODGERS-GRAY, T., OEHLMANN, J., SCHULTE-OEHLMANN, U., PAWLOWSKI, S., BAUNBECK, T., TURNER, A.P. AND TYLER C.R., 2004. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. *Aquatic Toxicology*, 66, 207-222.

JUNGMANN, D., LADEWIG, V., LUDWICHOWSKI, K.U., PETZSCH, P. AND NAGEL, R., 2004. Intersexuality in *Gammarus fossarum* KOCH - a common inducible phenomenon? *Archiv Fur Hydrobiologie*, 159 (4), 511-529.

KASHIAN, D. R., 2004. Toxaphene detoxification and acclimation in *Daphnia magna*: do cytochrome P-450 enzymes play a role? *Comparative Biochemistry and Physiology C (Toxicology & Pharmacology)*, 137, 3-63.

KIM, H.W., BATISTA, L.A., HOPPES, J.L., LEE, K.J. AND MYKLES, D.L., 2004a. A crustacean nitric oxide synthase expressed in nerve ganglia, Y-organ, gill and gonad of the tropical land crab, *Gecarcinus lateralis. Journal of Experimental Biology*, 207 (16), 2845-2857.

KIM, Y.J., SPALOVSKA-VALACHOVA, I., CHO, K.H., ZITNANOVA, I., PARK, Y., ADAMS, M.E. AND ZITNAN, D., 2004b. Corazonin receptor signalling in ecdysis initiation. *Proceedings of the National Academy of Sciences of the United States of America*, 101 (17), 6704-6709.

KIRKBRIDE-SMITH, A.E., BELL, H.A. AND EDWARDS, J.P., 2001. Effects of three vertebrate hormones on the growth, development and reproduction of the tomato moth, *Lacanobia oleracea* L. (Lepidoptera : Noctuidae). *Environmental Toxicology and Chemistry*, 20 (8), 1838-1845.

KOSTROUCH, Z., KOSTROUCHOVA, M. AND RALL, J.E., 2005. Steroid/thyroid hormone receptor genes in *Caenorhabditis elegans*. *Proceedings of the National Academy of Science USA*, 92, 156–159.

KUHN, A., MUNNS, W.R., CHAMPLIN, D., MCKINNEY, R., TAGLIABUE, M., SERBST, J. AND GLEASON, T., 2001. Evaluation of the efficacy of extrapolation population modelling to predict the dynamics of *Americamysis bahia* populations in the laboratory. *Environmental Toxicology and Chemistry*, 20 (1): 213-221.

KUSK, K.O. AND WOLLENBERGER, L., 2007. Towards an internationally harmonized test method for reproductive and developmental effects of endocrine disrupters in marine copepods. *Ecotoxicology*, 16, 183–195

LAFONT, R., 2000a. The endocrinology of invertebrates. *Ecotoxicology*, 9 (1-2), 41-57.

LAFONT, R., 2000b. Understanding insect endocrine systems: molecular approaches. *Entomologia Experimentalis Et Applicata*, 97 (2), 123-136.

LAUFER, H., AHL, J., ROTLLANT, G. AND BACLASKI, B., 2002. Evidence that ecdysteroids and methyl farnesoate control allometric growth and differentiation in a crustacean. *Insect Biochemistry and Molecular Biology*, 32 (2), 205-210.

LAVADO, R., BARBAGLIO, A., CANDIA CARNEVALI MD, AND PORTE, C., 2006. Steroid levels in crinoid echinoderms are altered by exposure to model endocrine disruptors. *Steroids*, 71, 489–497. Cited in Sugni *et al*., 2007.

LE CURIEUX-BELFOND, O., MOSLEMI, S., MATHIEU, M. AND SERALINI, G., 2001. Androgen metabolism in oyster *Crassostrea gigas*: Evidence for 17b-HSD activities and characterization of an aromatase-like activity inhibited by pharmacological compounds and a marine pollutant. *Journal of Steroid Biochemistry and Molecular Biology*, 78, 359-366.

LEBLANC, G.A., 2007. Crustacean endocrine toxicology: a review. *Ecotoxicology*, 16, 61-81.

LEBLANC, G.A., CAMBELL, P.M., DEN BESTEN, P., BROWN, R.P., CHANG, E., COATS, J., DEFUR, P.L., DHALDIALLA, T., EDWARDS, J., RIDDIFORD, L.M., SIMPSON, M., SNELL, T., THORNDYKE, M., MATSUMURA, F., 1999. The endocrinology of invertebrates. In: DeFur PL, Crane M, Ingersoll C, Tattersfield L (Eds) Endocrine disruption in invertebrates: endocrinology, testing, and assessment. SETAC. Press, Pensacola, FL, pp 23–106.

LEVOYE, A., MOUILLAC, B., RIVIERE, G., VIEAU, D., SALZET, M. AND BRETON, C., 2005. Cloning, expression and pharmacological characterization of a vasopressin-related receptor in an annelid, the leech *Theromyzon tessulatum*. *Journal of Endocrinology*, 184 (1), 277-289.

LICHT, O., JUNGMANN, D., LUDWICHOWSKI, K.-U., AND NAGEL, R., 2004. Long-term effects of fenoxycarb on two mayfly species in artificial indoor streams. *Environmental Toxicology and Chemistry*, 58, 246–255. Cited in Soin and Smagghe (2007).

MAIBECHE-COISNE, M., BOSCAMERIC, M., ARAGON, S., LAFONT, R. AND DAUPHIN-VILLEMANT, C., 2001. Molecular cloning of a novel crustacean member of the aldoketoreductase superfamily, differentially expressed in the antennal glands. *Biochemical and Biophysical Research Communications*, 282 (1), 314-320.

MALAUSA, T., SALLES, M., MARQUET, V., GUILLEMAUD, T., ALLA, S., MARION-POLL, F. AND LAPCHIN, L., 2006. Within-species variability of the response to 20-hydroxyeedysone in peach-potato aphid (*Myzus persicae* Sulzer). Journal of Insect Physiology, 52, 480-486.

MANIERE, G., VANHEMS, E., GAUTRON, F. AND DELBECQUE, J.P., 2002. Calcium inhibits ovarian steroidogenesis in the blowfly *Phormia regina. Journal of Endocrinology*, 173, (3):

MARCIAL, H.S., HAGIWARA, A. AND SNELL, T.W., 2003. Estrogenic compounds affect development of harpacticoid copepod *Tigriopus japonicus*. *Environmental Toxicology and Chemistry*, 22 (12), 3025-3030.

MARSHALL, D.J. AND RAJKUMAR, A., 2003. Imposex in the indigenous *Nassarius kraussianus* (Mollusca : Neogastropoda) from South African harbours. *Marine Pollution Bulletin*, 46, 1150-1155.

MCKENNEY Jr., C.L., 1998. Physiological dysfunction in estuarine mysids and larval decapods with chronic pesticide exposure. In: WELLS, P.G., LEE, K. AND BLAISE, C. *Microscale testing in aquatic toxicology: Advances, techniques, and practice.* CRC, Boca Raton, FL, USA, pp 465-476.

MCKENNEY Jr., C.L., HARRIS, P., NATES, S.F., CRIPE, G.M. AND HOGLUND, M.D., 1999. *Transgenerational effects of a juvenile hormone mimic on the estuarine mysid, Mysidopsis bahia (Crustacea: Mysidacea*). Abstracts, SETAC 20th Annual Meeting, Philadelphia, PA, USA, November 14-18, 1999, p 191.

MCKENNEY Jr., C.L., CRIPE, G.M., FOSS, S.S., TUBERTY, S.R. AND HOGLUND, M., 2004. Comparative embryonic and larval developmental responses of estuarine shrimp (*Palaemonetes pugio*) to the juvenile hormone agonist fenoxycarb. *Archives of Environmental Contamination and Toxicology*, 47 (4), 463-470.

MEREGALLI, G. AND OLLEVIER, F., 2001. Exposure of *Chironomus riparius* larvae to 17 alpha-ethinylestradiol: effects on survival and mouthpart deformities. *Science of the Total Environment*, 269 (1-3), 157-161.

MEREGALLI, G., PLUYMERS, L. AND OLLEVIER, F., 2001. Induction of mouthpart deformities in *Chironomus riparius* larvae exposed to 4-n-nonylphenol. *Environmental Pollution*, 111 (2), 241-246.

MOSCHINO, V. AND MARIN, M.G., 2002. Spermiotoxicity and embryotoxicity of triphenyl tin in the sea urchin *Paracentrotus lividus* Lmk. *Applied Organometallic Chemistry*, 16 (4), 175-181.

MOTOLA, D.L., CUMMINS, C.L., ROTTIERS, V., SHARMA, K.K., LI, T.T., LI, Y., SUIONO-POWELL, K., XU, H.E., AUCHUS, R.J., ANTEBI, A. AND MANGELSDORF, D.J., 2006. Identification of ligands for DAF-12 that govern dauer formation and reproduction in *C. elegans*. Cell 124:1209–1223

MU, X.Y., RIDER, C.V., HWANG, G.S., HOY, H. AND LEBLANC, G.A., 2005. Covert signal disruption: Anti-ecdysteroidal activity of bisphenol A involves cross talk between signalling pathways. *Environmental Toxicology and Chemistry*, 24 (1), 146-152.

NICE, H., MORRITT, D., CRANE, M. AND THORNDYKE, M., 2003. Long-term and transgenerational effects of nonylphenol exposure at a key stage in the development of

Crassostrea gigas. Possible endocrine disruption? *Marine Ecology Progress Series*, 256, 293-300.

NOVELLI, A.A., ARGESE, E., TAGLIAPIETRA, D., BETTIOL, C. AND GHIRARDINI, A.V., 2002. Toxicity of tributyl tin and triphenyl tin to early life-stages of *Paracentrotus lividus* (Echinodermata : Echinoidea). *Environmental Toxicology and Chemistry*, 21 (4), 859-864.

OBERDORSTER, E. AND CHEEK, A.O., 2001. Gender benders at the beach: Endocrine disruption in marine and estuarine organisms. *Environmental Toxicology and Chemistry*, 20 (1), 23-36.

OBERDORSTER, E. AND MCCLELLAN-GREEN, P., 2002. Mechanisms of imposex induction in the mud snail *Ilyanassa obsoleta*: TBT as a neurotoxin and aromatase inhibitor. *Marine Environmental Research*, 54, 715-718.

OBERDORSTER, E., RICE, C.D. AND IRWIN, L.K. 2000. Purification of vitellin from grass shrimp *Palaemonetes pugio*, generation of monoclonal antibodies, and validation for the detection of lipovitellin in Crustacea. *Comparative Biochemistry and Physiology C (Toxicology and Pharmacology*), 127 (2), 199-207.

OECD (1998) Daphnia magna reproduction test. OECD guidelines for testing of chemicals, 211. OECD: Paris.

OECD (2004a) OECD guidelines for the testing of chemicals, sediment-water chironomid toxicity test using spiked sediment, OECD guidelines for testing of chemicals, 218, OECD: Paris.

OECD (2004b) OECD Guidelines for the testing of chemicals, sediment-water chironomid toxicity test using spiked water. OECD guidelines for testing of chemicals, 219, OECD: Paris.

OECD, 2006. Detailed Review Paper on aquatic arthropods in life cycle toxicity tests with emphasis on developmental, reproductive and endocrine disruptive effects. OECD Environment Health and Safety Publications Series on Testing and Assessment No. 55. OECD: Paris.

OEHLMANN, J. AND SCHULTE-OEHLMANN, U., 2003. Endocrine disruption in invertebrates. *Pure and Applied Chemistry*, 75 (11-12), 2207-2218.

OEHLMANN, J., SCHULTE-OEHLMANN, U., TILLMANN, M. AND MARKERT, B., 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part 1: Bisphenol A and octylphenol as xenoestrogens. *Ecotoxicology*, 9, 383-397.

OETKEN, M., BACHMANN, J., SCHULTE-OEHLMANN, U. AND OEHLMANN, J., 2004. Evidence for endocrine disruption in invertebrates. *International Review of Cytology - A Survey of Cell Biology*, 236, 1-44.

OHJI, M., ARAI, T. AND MIYAZAKI, N., 2003. Biological effects of tributyl tin exposure on the caprellid amphipod, *Caprella danilevskii. Journal of the Marine Biological Association of the United Kingdom*, 83 (1), 111-117.

OKUMURA, T. AND AIDA, K., 2000. Fluctuations in hemolymph ecdysteroid levels during the reproductive and non-reproductive moult cycles in the giant freshwater prawn *Macrobrachium rosenbergii. Fisheries Science*, 66 (5), 876-883.

OKUMURA, T. AND AIDA, K., 2001. Effects of bilateral eyestalk ablation on moulting and ovarian development in the giant freshwater prawn, *Macrobrachium rosenbergii*. *Fisheries Science*, 67 (6): 1125-1135.

OKUMURA, T., KAMBA, M., SONOBE, H. AND AIDA, K., 2003. In vitro secretion of ecdysteroid by Y-organ during moult cycle and evidence for secretion of 3-dehydroecdysone in the giant freshwater prawn, *Macrobrachium rosenbergii* (Crustacea : Decapoda : Caridea). *Invertebrate Reproduction & Development*, 44 (1), 1-8.

OLMSTEAD, A.W. AND LEBLANC, G.A., 2000. Effects of endocrine-active chemicals on the development of sex characteristics of *Daphnia magna. Environmental Toxicology and Chemistry*, 19 (8), 2107-2113.

OLMSTEAD, A.W. AND LEBLANC, G.A., 2001. Low exposure concentration effects of methoprene on endocrine-regulated processes in the crustacean *Daphnia magna*. *Toxicological Sciences*, 62 (2), 268-273.

OLMSTEAD, A.W. AND LEBLANC, G.A., 2003. Insecticidal juvenile hormone analogues stimulate the production of male offspring in the crustacean *Daphnia magna*. *Environmental Health Perspectives*, 111 (7), 919-924.

PARK, Y., FILIPPOV, V., GILL, S.S. AND ADAMS, M.E., 2002. Deletion of the ecdysis-triggering hormone gene leads to lethal ecdysis deficiency. *Development*, 129 (2), 493-503.

PASCOE, D., CARROLL, K., KARNTANUT, W. AND WATTS, M.M., 2002. Toxicity of 17 alpha-ethinylestradiol and bisphenol A to the freshwater cnidarian *Hydra vulgaris*. *Archives of Environmental Contamination and Toxicology* 43, 56-63.

PATRICOLO, E., MANSUETO, C., D'AGATI, P. AND PELLERITO, L., 2001. Organometallic complexes with biological molecules: XVI. Endocrine disruption effects of tributyl tin (IV) chloride on metamorphosis of the ascidian larva. *Applied Organometallic Chemistry*, 15 (11), 916-923.

PINDER, L.C.V. AND POTTINGER, T.G., 1999. *Endocrine function in aquatic invertebrates and evidence for disruption by environmental pollutants*. Centre for Ecology and Hydrology, Natural Environmental Research Council, Ambleside

QUINN, B., GAGNE, F., COSTELLO, M., MCKENZIE, C., WILSON, J. AND MOTHERSILL, C., 2004. The endocrine disrupting effect of municipal effluent on the zebra mussel *(Dreissena polymorpha)*. *Aquatic Toxicology*, 66, 279-292.

QUINTELA, M., BARREIRO, R. AND RUIZ, J.M., 2000. The use of *Nucella lapillus* (L.) transplanted in cages to monitor tributyltin (TBT) pollution. Science of the Total Environment, 247, 227-237.

RADIX, P., SEVERIN, G., SCHRAMM, K.W. AND KETTRUP, A., 2002. Reproduction disturbances of *Brachionus calyciflorus* (rotifer) for the screening of environmental endocrine disrupters. *Chemosphere*, 47 (10), 1097-1101.

REDDY, P.R., NAGARAJU, G.P.C. AND REDDY, P.S., 2004. Involvement of methyl farnesoate in the regulation of moulting and reproduction in the freshwater crab *Oziotelphusa senex senex*. *Journal of Crustacean Biology*, 24 (3), 511-515.

REWITZ, K., STYRISHAVE, B. AND ANDERSEN, O., 2003. CYP330A1 and CYP4C39 enzymes in the shore crab *Carcinus maenas*: sequence and expression regulation by ecdysteroids and xenobiotics. *Biochemical and Biophysical Research Communications*, 310 (2), 252-260.

RICHARD, D.S., WATKINS, N.L., SERAFIN, R.B. AND GILBERT, L.I., 1998. Ecdysteroids regulate yolk protein uptake by *Drosophila melanogaster* oocytes. *Journal of Insect Physiology*, 44 (7-8), 637-644

RICHARD, D.S., JONES, J.M., BARBARITO, M.R., CERULA, S., DETWEILER, J.P., FISHER, S.J., BRANNIGAN, D.M. AND SCHESWOHL, D.M., 2001. Vitellogenesis in diapausing and mutant *Drosophila melanogaster*: further evidence for the relative roles of ecdysteroids and juvenile hormones. *Journal of Insect Physiology*, 47 (8), 905-913.

RIFFESER, M. AND HOCK, B., 2002. Vitellogenin levels in mussel Hhemolymph – A suitable biomarker for the exposure to estrogens? *Comparative Biochemistry and Physiology* C, 132, 75-84.

ROAST, S.D., THOMPSON, R.S., WIDDOWS, J. AND JONES, M.B., 1998. Mysids and environmental monitoring: A case for their use in estuaries. *Marine and Freshwater Research*, 49, 827–832.

RODGERS-GRAY, T.P., SMITH, J.E., ASHCROFT, A.E., ISAAC, R.E. AND DUNN, A.M., 2004. Mechanisms of parasite-induced sex reversal in *Gammarus duebeni*. *International Journal for Parasitology,* 34 (6), 747-753.

ROEPKE, T.A., SNYDER, M.J. AND CHERR, G.N., 2005. Estradiol and endocrine disrupting compounds adversely affect development of sea urchin embryos at environmentally relevant concentrations. *Aquatic Toxicology*, 71 (2), 155-173.

RUPPERT, E. AND BARNES, R., 1994. *Invertebrate zoology* (6th Edition), Saunders College Publishing.

SCHULTE-OEHLMANN, U., TILLMANN, M., MARKERT, B., OEHLMANN, J., WATERMANN, B. AND SCHERF, S., 2000. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part II: Triphenyl tin as a xenoandrogen. *Ecotoxicology*, 9, 399-412.

SNYDER, M.J. AND MULDER, E.P., 2001. Environmental endocrine disruption in decapod crustacean larvae: hormone titers, cytochrome P450, and stress protein responses to heptachlor exposure. *Aquatic Toxicology*, 55 (3-4), 177-190.

SOCHA, R., SULA, J. AND KODRIK, D., 2004. Wing morph-related differences in developmental pattern of accessory gland proteins in adult males of *Pyrrhocoris apterus* (L.) and their endocrine control. *Journal of Insect Physiology*, 50 (10), 893-901.

SOIN, T. AND SMAGGHE, G., 2007. Endocrine disruption in aquatic insects: a review. *Ecotoxicology*, 16, 83–93.

STRAND, J. AND JACOBSEN, J.A., 2002. Imposex in two sublittoral neogastropods from the Kattegat and Skagerrak: the common whelk *Buccinum undatum* and the red whelk *Neptunea antique*. Marine Ecology-Progress Series, 244, 171-177.

SUGNI, M., MOZZI, D., BARBAGLIO, A., BONASORO, F. AND CARNEVALI, M.D.C., 2007. Endocrine disrupting compounds and echinoderms: new ecotoxicological sentinels for the marine ecosystem. *Ecotoxicology*, 16, 95-108.

SUNDELIN, B., RYK, C. AND MALMBERG, G., 2000. Effects on the sexual maturation of the sediment-living amphipod *Monoporeia affinis*. *Environmental Toxicology*, 15 (5), 518-526.

TADA, M. AND HATAKEYAMA, S., 2000. Chronic effects of an insecticide, fenobucarb, on the larvae of two mayflies, *Epeorus latifolium* and *Baetis thermicus*, in model streams. *Ecotoxicology*, 9 (3), 187-195.

TAENZLER, V., BRUNS, E., DORGERLOH, M., PFEIFLE, V. AND WELTJE, L., 2007. Chironomids: suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. *Ecotoxicology*, 16, 221–230.

TAKAHASHI, T., ARAKI, A., NOMURA, Y., KOGA, M. AND ARIZONO, K., 2000. The occurrence of dual-gender imposer in Japanese freshwater crab. *Journal of Health Science*, 46 (5), 376-379.

TANEGA, C., RADMAN, D.P., FLOWERS, B., STERBA, T. AND WAGNER, G.F., 2004. Evidence for stanniocalcin and a related receptor in annelids. *Peptides*, 25 (10), 1671-1679.

TARRANT, A.M., 2007. Hormonal signaling in cnidarians: do we understand the pathways well enough to know whether they are being disrupted? *Ecotoxicology*, 16, 5-13.

TARRANT, A.M., BLOMQUIST, C.H., LIMA, P.H., ATKINSON, M.J. AND ATKINSON, S., 2003. Metabolism of estrogens and androgens by scleractinian corals. *Comparative Biochemistry and Physiology B (Biochemistry & Molecular Biology)*, 136 (3), 473-485.

TARRANT, A.M., ATKINSON, M.J. AND ATKINSON, S., 2004. Effects of steroidal estrogens on coral growth and reproduction. *Marine Ecology Progress Series*, 269, 121-129.

TATARAZAKO, N. AND ODA, S., 2007. The water flea *Daphnia magna* (Crustacea, Cladocera) as a test species for screening and evaluation of chemicals with endocrine disrupting effects on crustaceans. *Ecotoxicology*, 16, 197–203.

TATARAZAKO, N., TAKAO, Y., KISHI, K., ONIKURA, N., ARIZONO, K. AND IGUCHI, T., 2002. Styrene dimers and trimers affect reproduction of daphnid (*Ceriodaphnia dubia*). *Chemosphere*, 48 (6), 597-601.

THORNTON, J., NEED, E. AND CREWS, D., 2003. Resurrecting the ancestral steroid receptor: Ancient origin of estrogen signalling. *Science*, 301, 1714-1717.

TILLMANN, M., SCHULTE-OEHLMANN, U., DUFT, M., MARKERT, B. AND OEHLMANN, J., 2001. Effects of endocrine disruptors on prosobranch snails (Mollusca: Gastropoda) in the laboratory. Part III: Cyproterone acetate and vinclozolin as anti-androgens. *Ecotoxicology*, 10, 373-388.

TOMINAGA, N., URA, K., KAWAKAMI, M., KAWAGUCHI, T., KOHRA, S., MITSUI, Y., IGUCHI, T. AND ARIZONO, K., 2003. *Caenorhabditis elegans* responses to specific steroid hormones. *Journal of Health Science,* 49 (1): 28-33.

TUBERTY, S.R., NATES, S.F. AND MCKENNEY, C.L., 2000. Polyclonal antisera against estuarine crustacean vitellins: a molecular approach to reproductive endocrinology and toxicology. *American Zoologist*, 40, 1239-1239.

URA, K., KAI, T., SAKATA, S., IGUCHI, T. AND ARIZONO, K., 2002. Aquatic acute toxicity testing using the nematode *Caenorhabditis elegans*. *Journal of Health Science*, 48 (6), 583-586.

US ENVIRONMENTAL PROTECTION AGENCY, 1995. *Short-term methods for estimating the chronic toxicity of effluents and surface waters to marine and estuarine organisms* (3rd Edition). EPA-600-4-91-002: Cincinnati, USA.

US ENVIRONMENTAL PROTECTION AGENCY, 2002. *Methods for measuring the acute toxicity of effluents and receiving waters to frehwater and marine organisms* (5th Edition). EPA-821-R-02-012: Washington DC, USA.

USEPA (2000) Methods for measuring the toxicity and bioaccumulation of sediment associated contaminants with freshwater invertebrates, 2nd Edn. EPA 600/R-99/064, USEPA: Washington DC, USA.

VANDENBERGH, G.F., ADRIAENS, D., VERSLYCKE, T. AND JANSSEN, C.R., 2003. Effects of 17 alpha-ethinylestradiol on sexual development of the amphipod *Hyalella azteca* *Ecotoxicology and Environmental Safety*, 54, 216-222.

VERMEULEN, A.C., LIBERLOO, G., DUMONT, P., OLLEVIER, F. AND GODDEERIS, B., 2000. Exposure of Chironomus riparius larvae (diptera) to lead, mercury and beta-sitosterol: effects on mouthpart deformation and moulting. Chemosphere, 41 (10), 1581-1591.

VERSLYCKE, T., 2003. *Endocrine disruption in the estuarine invertebrate Neomysis integer (Crustacea: Mysidacea*). Unpublished PhD thesis. Ghent University.

VERSLYCKE, T., DE WASCH, K., DE BRABANDER, H.F. AND JANSSEN, C.R., 2002. Testosterone metabolism in the estuarine mysid *Neomysis integer* (Crustacea; Mysidacea): Identification of testosterone metabolites and endogenous vertebrate-type steroids. *General and Comparative Endocrinology*, 126 (2), 190-199

VERSLYCKE, T., POELMANS, S., DE WASCH, K., VERCAUTEREN, J., DEVOS, C., MOENS, L., SANDRA, P., DE BRABANDER, H.F. AND JANSSEN, C.R., 2003. Testosterone metabolism in the estuarine mysid *Neomysis integer* (Crustacea; Mysidacea) following tributyl tin exposure. *Environmental Toxicology and Chemistry*, 22 (9), 2030-2036.

VERSLYCKE, T., FOCKEDEY, N., MCKENNEY JR., C.L., ROAST, S.D., JONES, M.B., MEES, J. AND JANSSEN, C.R., 2004. Mysid crustaceans as potential test organisms for the evaluation of environmental endocrine disruption: A review. *Environmental Toxicology and Chemistry*, 23 (5), 1219-1234.

VERSLYCKE, T., GHEKIERE, A., RAIMONDO, S. AND JANSSEN, C., 2007. Mysid crustaceans as standard models for the screening and testing of endocrine-disrupting chemicals. *Ecotoxicology*, 16, 205-219.

VOLZ, D.C. AND CHANDLER, G.T., 2004. An enzyme-linked immunosorbent assay for lipovitellin quantification in copepods: A screening tool for endocrine toxicity. *Environmental Toxicology and Chemistry*, 23 (2), 298-305.

WANG, C. AND CROLL, R., 2003. Effects of sex steriods on in vitro gamete release in the sea scallop, *Placopecten megellanicus*. *Invertebrate Reproduction and Development,* 44, 2-3, 89-100.

WANG, C. AND CROLL, R., 2004. Effects of sex steriods on gonadal development and gender determination in the sea scallop, *Placopecten megellanicus. Aquaculture,* 238, 483-498.

WATTS, M.M., PASCOE, D. AND CARROLL, K., 2001. Chronic exposure to 17 alpha-ethinylestradiol and bisphenol A-effects on development and reproduction in the freshwater invertebrate *Chironomus riparius* (Diptera: Chironomidae). *Ecotoxicology And Environmental Safety,* 54, 207-215.

WATTS, M.M., PASCOE, D. AND CARROLL, K., 2003. Exposure to 17 alpha-ethinylestradiol and bisphenol A-effects on larval moulting and mouthpart structure of *Chironomus riparius*. *Ecotoxicology and Environmental Safety*, 54 (2), 207-215.

WOLLENBERGER, L., DINAN, L. AND BREITHOLTZ, M., 2005. Brominated flame retardants: Activities in a crustacean development test and in an ecdysteroid screening assay. *Environmental Toxicology and Chemistry*, 24 (2), 400-407.

ZERA, A.J. AND BOTTSFORD, J., 2001. The endocrine-genetic basis of life-history variation: The relationship between the ecdysteroid titer and morph-specific reproduction in the wing-polymorphic cricket *Gryllus firmus.* *Evolution*, 55 (3), 538-549.

ZERA, A.J. AND ZHAO, Z.W., 2003. Morph-dependent fatty acid oxidation in a wing-polymorphic cricket: implications for the trade-off between dispersal and reproduction. *Journal of Insect Physiology*, 49 (10), 933-943.

ZERA, A.J. AND ZHAO, Z.W., 2004. Effect of a juvenile hormone analogue on lipid metabolism in a wing-polymorphic cricket: Implications for the endocrine-biochemical bases of life-history trade-offs. *Physiological and Biochemical Zoology*, 77 (2), 255-266.

ZITNAN, D., HOLLAR, L., SPALOVSKA, I., TAKAC, P., ZITNANOVA, I., GILL, S.S. AND ADAMS, M.E., 2002. Molecular cloning and function of ecdysis-triggering hormones in the silkworm *Bombyx mori. Journal of Experimental Biology*, 205 (22), 3459-3473.

ZITNAN, D., ZITNANOVA, I., SPALOVSKA, I., TAKAC, P., PARK, Y. AND ADAMS, M.E., 2003. Conservation of ecdysis-triggering hormone signalling in insects. *Journal of Experimental Biology*, 206 (8), 1275-1289.

ZOU, E., 2005. Impact of xenobiotics on crustacean molting: the invisible endocrine disruption. *Integrated Comparative Biology*, 45, 33–38.

ZULKOSKY, A.M., FERGUSON, P.L. AND MCELROY, A.E., 2002. Effects of sewage-impacted sediment on reproduction in the benthic crustacean *Leptocheirus plumulosus*. *Marine Environmental Research*, 54 (3-5), 615-619.

List of abbreviations

20-E	20-hydroxyecdysone
A	atrazine
ASTM	American Standards for Testing and Materials
BaP	benzo(a)pyrene
BDE-28	brominated diphenyl ether – 28
BDE-47	brominated diphenyl ether – 47
BDE-99	brominated diphenyl ether – 99
BDE-100	brominated diphenyl ether – 100
BFR	brominated flame retardant
bis-A	bisphenol A
CMP	cypris major protein
DEHP	di(ethyl-hexyl)phthalate
DES	diethylstilbestrol
E1	oestrone
E2	17 ß-oestradiol
E3	oestriol
ED	endocrine disruption
EDC	endocrine disrupting chemical
EE2	ethinyloestradiol
ELH	egg-laying hormone
GH	growth hormone
GRH	gonadotrophin-releasing hormone
MF	methyl farnesoate
MIH	moult-inhibiting hormone
NO	nitric oxide
NP	nonylphenol (also 4NP, 4-nonylphenol)
NPEO	nonylphenol ethoxylate
OECD	Organisation of Economic Cooperation and Development
p-t-OP	p-t-octyl phenol
STW	sewage treatment works
TBBPA	tetrabromobisphenol A
TBP	tribromophenol

TBT	tributyl tin
TBT-Cl	tributyl tin chloride
US EPA	United States Environmental Protection Agency
Vtg	vitellogenin

Appendix 1:

EXECUTIVE SUMMARY (Environment Agency, 1999)

Endocrinology of invertebrates

2. The current state of understanding of the endocrinology of the major invertebrate groups has been summarised. It is clear that utilisation of hormones to control and coordinate biochemical, physiological and behavioural processes is common to all major invertebrate taxa. Neuropeptide signalling mechanisms which utilise the peptide products of specialised neurosecretory cells are the predominant effectors among the endocrine systems so far characterised in invertebrates. However, non-peptide endocrine messengers are also important in many groups. Both of these systems are potentially “at risk” from interference by disruptive contaminants.

3. Among the aquatic invertebrate taxa the endocrine systems of the two major arthropod classes, insects and crustaceans, are best documented. In addition to peptide hormones, insects utilise homosesquiterpenoid epoxides (the juvenile hormones) and ecdysteroids (ecdysone, 20-hydroxyecdysone), neither of which are found in vertebrates. A range of vertebrate-type steroids (androgens, estrogens, progestogens, corticosteroids) have been detected in insects but it remains to be proven whether these have a functional role. A similar situation occurs in the crustacea which possess a wide range of peptide hormones and also utilise ecdysteroids. In addition to ecdysteroids, the non-peptide methyl farnesoate acts as a hormone in crustacea. As is the case with insects, although vertebrate-type steroids are detected in crustacean tissues their functional significance is yet to be confirmed.

4. Although not as well documented as for arthropods, the endocrine systems of the molluscs have been extensively studied. Molluscs utilise a wide range of peptide hormones to control and coordinate major processes. In contrast to the arthropods, ecdysteroids do not appear to play an important role in molluscs. The presence of vertebrate-type steroids has been reported for a number of molluscan species and in some cases the evidence that these steroids play a functional role is strong.

5. Evidence for a significant role of vertebrate-type steroids is strongest within the echinoderms. In addition to peptide signalling and the use of the purine 1-methyladenine in the later stages of oocyte development, there is considerable published evidence suggesting that vertebrate-type steroids are synthesised by echinoderms and may have a role in the control of growth and reproduction.

6. For the remaining groups considered within the review (the Coelenterata, Porifera, Acoelomata, Aschelminthes and Annelida), there are varying degrees of understanding of endocrine-type processes and isolated reports of steroid synthesising activity.

7. If concerns regarding possible avenues for endocrine disruption in invertebrates are focused on non-peptide systems, it is clear that all invertebrate groups must be considered *at risk* or potentially susceptible to interference at a sub-lethal level by chemicals in the aquatic environment. However, given the complex and multi-functional nature of the role of those nonpeptide hormones whose functions are best understood in invertebrates, the likely impact of interference at any particular locus within the endocrine system is difficult to predict. It is probable that effects of disruption will encompass reproduction, moulting, feeding, and behaviour.

Ecotoxicological testing

9. The development of bioassays and biomarkers to assess endocrine disruption in aquatic invertebrates is, in principle, feasible. However, there appears to be no imperative for the development of wholly novel test systems. Uncertainty regarding the function of the endocrine system in many invertebrate species and a lack of understanding of the identity and possible sites of action of endocrine disrupting chemicals precludes the development and application of cellular and subcellular screens for endocrine disrupting activity in invertebrates. The greatest benefits may be obtained by modifying the endpoints of, or carrying out more detailed assessments in, existing test protocols.

10. Invertebrate test organisms provide the ability to monitor entire life cycles more readily than is the case for vertebrates, offering the prospect that integrative effects of exposure to potential endocrine disrupters can be detected more readily than is the case for vertebrates. Given the difficulties being encountered in relating cellular-level, or receptor-level, effects to whole-animal consequences by those working with endocrine disrupters in vertebrates, this may be an advantage, not a disadvantage. Clearly, invertebrates with short generation times offer the possibility of examining transgenerational and population level effects.

11. Caution should be employed when ascribing adverse effects of contaminant exposure to disruption of endocrine processes. Relatively few cases of contaminant effects on vertebrate reproductive processes can be confidently ascribed to endocrine disruption and the term *endocrine disruption* has been applied to cases where the biological/mechanistic basis for such effects is not established. This concern is exacerbated in the case of invertebrates where, in many cases, physiological and endocrine processes which may be affected are less well understood than is the case for many vertebrate species.

Evidence for endocrine disrupting effects

12. A range of compounds with known endocrine disrupting activity in invertebrates have been deliberately introduced into the environment and these frequently have been shown to have impacts on non-target species. Examples are the pesticides tebufenozide, a potent ecdysone agonist, and methoprene which is a juvenile hormone analogue. Tebufenozide is highly toxic to Cladocera, for which there is no apparent safe concentration, but surprisingly not to Copepoda. Methoprene is used in mosquito control and adversely affects reproductive performance of the crustacean *Mysidopsis bahia* at very low concentrations, probably through interference with the endogenous endocrine system. Effects have also been observed in a variety of other Crustacea but, as with tebufenozide, susceptibility varies markedly, between taxa and life stages.

13. A number of other pesticides, not specifically designed to interact with invertebrate hormonal systems, also have effects, at low concentrations, that are suspected in some cases to be mediated through endocrine disruption. These include the herbicides atrazine, diquat and MCPA, the insecticides DDT and its derivatives, endosulfan and the biocide tributyltin (TBT).

14. Tributyltin is the best known endocrine disrupter in invertebrates, having been identified as the agent responsible for global declines in populations of several molluscan species, through interference with reproduction. TBT is believed to inhibit the P-450-dependent aromatase responsible for conversion of testosterone to estradiol-17b.

15. The most commonly described effect of TBT exposure, termed imposex, is the imposition of male reproductive organs on the female of neogastropods. In some

species, such as *Nucella lapillus*, this leads to the blocking of the female genital tract and consequent infertility. In some other closely related species infertility does not result from imposex, either because the underlying morphology prevents the female genital pore becoming blocked by the enlarged male organs or because the vulva elongates along with the growth of the penis and vas deferens. Worldwide, imposex has been reported to occur in 70 species of mollusc. There are few reports of imposex among freshwater molluscs but it has been reported from a tropical freshwater species, *Marisa cornuarietis,* and reduced egg laying was noted in *Biomphalaria glabrata* when exposed to a very low concentration of TBT.

16. Sterilisation by less obvious means may occur in other molluscs when exposed to TBT. For example, inhibition of larval production in the oyster *Ostrea edulis* may have been the result of hormonal retardation of the normal change from male to female during the reproductive cycle.

17. In the periwinkle *Littorina littorea,* TBT exposure gives rise to development of the intersex condition through the development of male features in the female genital tract or supplanting of the female sex organs by those of the male. This is distinct from the imposex response shown by many neogastropods, where the male organs are superimposed on those of the female. A gradation of levels of response are evident, ranging from incomplete closure of the female genital tract to development of a seminal groove and small penis. No other signs of sex change or evidence of spermatogenesis have been identified.

18. Intersexuality is common in a number of crustaceans and is often associated with parasitic castration. However, in harpacticoid copepods intersexuality is extremely rare. Unusually, a very high proportion of several species of harpacticoid have been found in the neighbourhood of the long-sea sewage outfall in the North Sea near Edinburgh. A causal relationship between this phenomenon and some form of chemical pollution is deemed to be a high possibility in this case. No effects were evident on community structure and all meiobenthic samples showed a high diversity of copepods. No traces of parasitism were detected. Attempts to induce the effect in the laboratory using TBT were not successful.

19. A number of metals, notably cadmium, and PCBs are suspected of disrupting endocrine function in invertebrates. Cadmium and PCBs cause aberrations in the early development of the sea stars, *Asterias rubens* and *Patiria miniata* and the sea urchin *Strongylocentrus intermedius.* In male and female sea stars, these chemicals cause significant reductions in the levels of progesterone and testosterone in the pyloric caeca and after prolonged exposure to PCBs, elevated levels of testosterone were found in the testes and ovaries of sea stars. PCBs have also been shown to affect ovary growth and both cadmium and PCBs are believed to interfere with hormonal control of reproduction by steroids.

20. Colour changes in some crustaceans are regulated by pigment dispersing and pigment concentrating hormones. Both cadmium and a PCB, Aroclor 1242, inhibit the release of the black pigment dispersing hormone in the crab *Uca pugilator.*

21. Exposure of *D magna* to even very low concentrations of cadmium affects reproduction, apparently through interference with the endocytotic uptake of yolk by the oocytes. At slightly higher concentrations the intermoult period was also extended. Selenium has also been shown to inhibit or delay ecdysis. The role of ecdysteroids in crustacean reproduction is not properly understood, but it seems that primary and secondary vitellogenesis are under ecdysteroid control, while 20-hydroxyecdysterone is well documented as having the major positive influence on the moulting cycle.

22. It has been hypothesised that the flexible cladoceran sex ratio may be more easily influenced by hormone-like xenobiotics than that of obligate sexual species, leading to a prediction that the maximum frequency of males in any one year would have been

higher before 1945. Data for Lake Mendota for 1895, 1975 and 1991 show a "dramatic" decrease in the frequency of males for two *Daphnia* species with time.

23. The estrogenic insecticide endosulfan and the synthetic estrogen diethylstilbestrol (DES) have no effect on sex differentiation in *Daphnia magna* but do influence reproductive success in this species. Chronic levels of exposure to DES also resulted in reduced moulting frequency.

24. The estrogenic alkylphenol, 4-nonylphenol, apparently reduces the rate of elimination of testosterone in *D. magna*, leading to accumulation of androgenic products and reduced fecundity of females. In recent research, in which *D magna* were exposed to p-*tert*pentylphenol (PTP), some females showed conspicuous malformations of the carapace from which it appeared that they had undergone a form of external masculinization.

25. Production of eggs and females by *Daphnia* were both affected by exposure to nonylphenol, though production of males was less sensitive. Similar effects also arose when females were exposed to a toxic strain of *Microcystis* (Cyanobacteria).

26. Plants produce chemicals known as phytoecdysteroids that are structurally very similar to the ecdysteroids of insects and crustaceans. These are very soluble in water and can compete with 20-hydroxyecdysone and so interfere with the ecdysteroid hormone system. Bioassays have demonstrated that many phytoecdysteroids exhibit moulting hormone activity in insects. Dragonflies exposed to paper- and pulp-mill (and tannery) effluent showed a shortened time to first moult and arrested moulting in the larvae, leading to a suggestion that the effluents contained juvenile hormone mimics.

27. Deformities in larvae of Chironomidae are associated with sediment contamination by heavy metals, phthalates and organochlorine pesticides and it is possible that these may result from disruption of hormone metabolism. If this is the case they could provide useful endpoints for laboratory bioassay. More research is required in this field.

28. Endocrine mechanisms are responsible for organizing some types of invertebrate behaviour and further research in this area could also provide useful bioassay endpoints.

Conclusions and Recommendations

29. Because the use of hormones to control and coordinate physiological and behavioural processes is common to all major invertebrate taxa, all invertebrate groups must be considered “at risk” or potentially susceptible to interference at a sub-lethal level by endocrine disrupting chemicals.

30. The detection of effects of exposure to contaminants in both natural and laboratory populations of invertebrates, and the assessment of the ability of chemicals to interfere with endocrine-dependent processes (growth, reproduction, behaviour), does not require a detailed understanding of the endocrinology of the organism concerned.

31. However, an understanding of the endocrinology of relevant organisms is important if the mechanisms by which environmental contaminants elicit effects are to be accurately attributed. Supporting work on mechanisms is therefore important to develop definitive evidence for which chemicals are invertebrate endocrine disruptors.

32. The major research need in the first instance is for field surveys to establish whether there is any evidence for endocrine disruption in individuals or populations. Systematic biological monitoring is needed in situations where chemicals with endocrine disrupting capability are most likely to be found, notably in rivers

downstream of sewage or industrial effluents and in the neighbourhood of sewage outfalls in the marine environment.

33. Current biological water quality monitoring should then be extended to take into account appropriate indicators of endocrine disruption.

34. To do this effectively, it will be necessary to identify a range of "sentinel" organisms together with appropriate endpoints/indicators of endocrine-disrupting activity.

35. In the freshwater environment, sentinels, and subjects chosen for bioassay, should include representatives of the Annelida, Mollusca, Crustacea, and Insecta and for the marine environment Coelenterata, Annelida, Mollusca, Crustacea and Echinodermata should be represented.

36. Many existing invertebrate toxicity-testing protocols provide data on the major processes that might be impacted by endocrine disrupting chemicals, to a greater extent than is the case for vertebrates.

37. It is likely that thorough testing with existing invertebrate protocols would detect chemicals that exert adverse effects via the endocrine system. However, existing protocols tend not to provide data on the mechanistic basis of observed toxic effects.

38. What must be determined is whether:

a: it is appropriate to instigate a test regime (or continue with existing test strategies) that identifies effects without necessarily identifying the route by which those effects occur, or

b: whether it is important to discriminate between compounds which act via the endocrine system and those which do not.

39. The former strategy involves the minimum of investment in new techniques and test methods; the latter strategy would require considerable investment in research to identify indicators of disruption in specific elements of the endocrine systems of a diverse range of invertebrate species.

40. The most appropriate use of research resources might encompass a dual track approach - continued testing with existing protocols which include response measures likely to detect effects of endocrine disruption in addition to other modes of toxicity, together with a research programme targeted at identifying the mechanisms underlying effects attributable to compounds suspected of endocrine disrupting capabilities.

Appendix 2:

Search terms used on the Web of Science

endocrin*	and	disrupt*	and	invert*
"	and	"	and	agapetus*
"	and	"	and	amphipod*
"	and	"	and	chironomid*
"	and	"	and	cladocer*
"	and	"	and	clistoronia*
"	and	"	and	coleoptera
"	and	"	and	daphni*
"	and	"	and	ephemeroptera
"	and	"	and	freshwater
"	and	"	and	hemipt*
"	and	"	and	hexagenia
"	and	"	and	insect
"	and	"	and	malacostraca*
"	and	"	and	mysid*
"	and	"	and	trichoptera
endocrin*	and	invert*	and	aquatic
"	and	"	and	biomarker
"	and	"	and	chemical*
"	and	"	and	endpoint
"	and	"	and	endpoint*
"	and	"	and	function
"	and	"	and	immune
"	and	"	and	immune*
"	and	"	and	land*
"	and	"	and	lethal*
"	and	"	and	moulting
"	and	"	and	moulting*
"	and	"	and	ovar*
"	and	"	and	styrene*
"	and	"	and	styrene*
"	and	"	and	toxi*
xenobio*	and	"		
ecdysteroid*	and	"		
estrogen*	and	"		
oestrogen*	and	"		
estradiol	and	"		
xenobio*	and	"		
edc	and	"		
edc	and	freshwater		
ecdysteroid*	and	freshwater		
ecdysteroid*	and	endpoint*		

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