Endocrine Function in Aquatic Invertebrates and Evidence for Disruption by Environmental Pollutants

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Statement of use
The purpose of this report is to identify the potential impacts that might occur in invertebrates from exposure to endocrine disrupting chemicals (EDCs). The report reviews the endocrinology of the invertebrate groups and presents evidence for endocrine disruption from laboratory and field investigations. The report is to be used by the Agency to:

- highlight the risk to invertebrates from EDCs, and the effects that could occur in groups particularly at risk.
- identify knowledge gaps that should be addressed to assess the "health" status of invertebrate populations
- inform Agency policy on the investigation, and control, of endocrine disrupting chemicals released to the environment.

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EXECUTIVE SUMMARY

Objectives

1. This report addresses five primary objectives:-

(i) to summarize the key elements of invertebrate endocrine systems;

(ii) to assess whether existing test systems are adequate for the detection of endocrine disruption in invertebrates, what new tests might be required, which species of invertebrates are most appropriate for such tests, what end-points should be measured and whether the same organisms can be used for both laboratory and environmental monitoring;

(iii) to review the published evidence for endocrine disruption in aquatic invertebrates (marine and freshwater; experimental and field studies) resulting from exposure to chemicals in the environment;

(iv) to summarize the relevant UK and European legislation impacting on the monitoring and management of freshwater ecosystems;

(v) to highlight the major gaps in knowledge and present an outline research programme to improve our understanding of the processes underpinning endocrine disruption in invertebrates.

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 Aat 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 non-peptide 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.

Regulatory Issues

8. Legislation and guidelines, relevant to the control of endocrine substances in the environment in Europe and North America, are reviewed.

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 end-points 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 Aendocrine 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 oestradiol-17β.

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 2 *Daphnia* species with time.

23. The oestrogenic insecticide endosulfan and the synthetic oestrogen 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 oestrogenic 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 end-points 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 end-points.

Research needs

29. An understanding of the endocrinology of relevant organisms is important if the mechanisms by which environmental contaminants elicit effects are to be accurately attributed. However, 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. Therefore, while undoubtedly important, research into the basic endocrinology of invertebrate species need not be considered a priority requirement. Existing research effort, which is driven by factors other than concerns arising from consideration of endocrine disruption, should be sustained and will continue to assist understanding within the field of invertebrate endocrine disruption.

30. It must be determined whether it is appropriate to instigate a test regime (or continue

with existing test strategies) which identifies effects without necessarily identifying the route by which those effects occur, or whether it is important to discriminate between compounds which act via the endocrine system and those which do not. 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. The most appropriate use of 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. An understanding of mechanisms may help to more accurately assess risks and shift patterns of chemical use to minimise future problems.

31. 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. Current biological water quality monitoring should be extended to take into account appropriate indicators of endocrine disruption. To do this effectively, it will be necessary to identify a range of "sentinel" organisms together with appropriate end-points/indicators of endocrine-disrupting activity. 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. It is not appropriate to include studies of the mechanisms of endocrine disruption in routine environmental management programmes; nonetheless, they should be supported in universities and research institutes. 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.

KEY WORDS

Invertebrates, steroid hormones, endocrine disruption, aquatic research, ecotoxicology, biomarkers

GLOSSARY / ABBREVIATIONS

20-HE	20-hydroxyecdysone
4-NP	4-nonylphenol
5-HT	5-hydroxytryptamine
ACTH	adrenocorticotropin
AGH	androgenic gland hormone
AKH	adipokinetic hormone
APHA	American Public Health Association
ASO	accessory sex organs
ASTM	American Society for Testing and Materials
BC	bag cells
BPDH	black pigment-dispersing hormone
CA	corpora allata
cAMP	cyclic adenosine monophosphate
CC	corpora cardiaca
CCAP	crustacean cardioactive peptide
CCK	cholecystokinin
CDCH	caudodorsal cell hormone
cGMP	cyclic guanosine monophosphate
CHH	crustacean hyperglycaemic hormone
CMF	coelomic maturation factor
CNS	central nervous system
CVC	cerebrovascular complex
DAH	dark-adapting distal retinal pigment hormone
DBH	dorsal body hormone
DDE	1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
DDT	2,2-bis(chlorophenyl)-1,1,1-trichloroethane
DES	diethylstilbestrol
DFB	diflubenzuron
DGC	dark green cell system
EA	Environment Agency (UK)
EC	European Commission
ECH	erythrophore concentrating hormone
ECLS	egg capsule laying substance
EH	eclosion hormone
ELH	egg laying hormone
EPA	Environmental Protection Agency (USA)
FMRFamide	Phe-Met-Arg-Phe-NH_2
GC-MS	gas chromatography-mass spectrometry
GIH	gonad-inhibiting hormone (=VIH)
GSH	gonad-stimulating hormone
GSS	gonad-stimulating substance
hCG	human chorionic gonadotropin
HPLC	high performance liquid chromatography
JH	juvenile hormone
LAH	light-adapting distal retinal pigment hormone
LCH	leukophore concentrating hormone (=WPCH)
LDB	laterodorsal bodies
LGC	light green cell system
LH	luteinising hormone
LHRH	luteinising hormone releasing hormone
MCPA	4-chloro-2-methyl phenoxyacetic acid
MDB	mediodorsal bodies
MDH	melanophore dispersing hormone
MF	methyl farnesoate
MFO	mixed function oxidases
MIF	melanocyte-stimulating hormone release-inhibiting factor
MIH	moult-inhibiting hormone
MIP	molluscan insulin-related peptide
MIS	maturation-inducing substance (1-methyladenine)
MOIH	mandibular organ-inhibiting hormone
MPF	maturation-promoting factor
MRCH	melanization and reddish coloration hormone
NDH	neurodepressing hormone
OECD	Organisation for Economic Cooperation and Development
PBAN	pheromone biosynthesis and activating neuropeptide
PCBs	polychlorinated biphenyls
PCP	pentachlorophenol
PRL	prolactin
PTP	p-ter-pentylphenol
PTTH	prothoracotropic hormone
RIA	radioimmunoasay
RNF	radial nerve factor
RPCH	red pigment-concentrating hormone
SEF	secretory end feet
SETAC	Society of Environmental Toxicology and Chemistry
SG	sinus gland
SIS	sodium influx stimulating peptide
SMF	sperm maturation factor
TBT	tributyltin
TOH	temporary ovarian hormone
VIH	vitellogenesis-inhibiting hormone (=GIH)
VSOH	vitellogenin-stimulating ovarian hormone
WPCH	white pigment-concentrating hormone (=LCH)
YC	yellow cells
YGC	yellow green cells

1. INTRODUCTION

Over the last five years, the international scientific community has grown increasingly concerned that exposure to low levels of anthropogenic chemicals may disturb hormone function in Man and other animals - so-called endocrine disruption - (e.g Colborn *et al*, 1996). Reproductive hormone-receptor systems appear to be particularly vulnerable. Indeed, changes in sperm counts, genital tract malformations, infertility and an increased frequency of mammary, prostate and testicular tumours, have all been reported (Sharpe and Skakkebaek, 1993; Colborn *et al*, 1996). Hormonal disturbances in wildlife include sex changes in riverine fish (Sumpter, 1995) and marine snails (Matthiessen and Gibbs, 1998), reproductive failure in birds (Spitzer *et al*., 1978), and abnormalities in the reproductive organs of alligators (Guillette *et al*., 1994). The endocrine and reproductive effects of pollutants are believed to be due to their ability to:-

(a) mimic, or alternatively anatagonise, the effects of hormones;
(b) alter the pattern of synthesis and metabolism of hormones;
(c) modify hormone receptor levels (Soto *et al*. 1995).

Chemicals, that are thought to be capable of disrupting the reproductive endocrine systems of animals, fall into the following categories:-

(i) Environmental oestrogens (oestrogen receptor mediated) (e.g. Methoxychlor, bisphenolic compounds).
(ii) Environmental antioestrogens (e.g. Dioxin, Endosulphan).
(iii) Environmental antiandrogens (e.g. Vinclozolin, DDE, Kraft mill effluent).
(iv) Toxicants that reduce steroid hormone levels (e.g. Fenarimol and other fungicides, endosulphan).
(v) Toxicants that affect reproduction primarily through effects on the CNS (e.g. dithiocarbamate pesticides, methanol).
(vi) Other toxicants that affect hormonal status (e.g. cadmium, benzidine-based dyes).

At present, the endocrine disrupters of greatest concern are those that, despite having widely diverse chemical structures, mimic oestrogen. They are referred to as oestrogenic xenobiotics or xenoestrogens. Xenoestrogens may be components of the diet or exposure may occur via direct uptake across the body surfaces.

In developing management strategies to address the potential problem of endocrine disruption, the responsible bodies (e.g environmental regulators; UK Environment Agency) are confronted with significant problems. There are numerous chemicals within the environment that may possess endocrine disrupting potential. Many of these chemicals have little in common structurally or in terms of their chemical properties (e.g. see Table 2 for the range of chemicals that may disrupt endocrine function in invertebrates). There is also considerable debate as to how chemical mixtures produce endocrine disrupting effects; do the components act additively, synergistically or antagonistically (Arnold *et al*., 1997; Ashby *et al*., 1997; Ramamoorthy *et al*., 1997)? Furthermore, there is still much to learn regarding routes of uptake, bioactivation and biotransformation, and excretion of endocrine disruptors.

A number of natural hormone-like chemicals also exist, the effects of which are far from fully understood. For example, phytoestrogens and mycoestrogens, natural products of plants and fungi, have been shown to bind more readily to oestrogen receptors than most xenoestrogens in *in vitro* assay systems, yet their role in endocrine disruption is obscure. It has been suggested that, in insects, consumption of phytoestrogens may result in alterations in sex ratio which ultimately reduce population size, thereby reducing herbivory.At present it is unknown how these natural chemicals might interact with xenooestrogens.

Invertebrates constitute ca. 95% of all animal species and are key components of all ecosystems. However, current ecotoxicological assessments may neglect the potential of chemicals to cause endocrine disruption in invertebrates. In most toxicity assessments, an invertebrate species (commonly, *Daphnia* sp.) is used to gain some insight into the potential toxicity of chemicals. These data are used as surrogates for diverse phyla that differ markedly in their vulnerability to pollutant toxicity (and presumably to endocrine disruption as well) due to very pronounced differences in biochemistry and physiology. As well as interspecies differences in susceptibility to endocrine disruption it is likely that there are also marked intraspecific differences in susceptibility between individuals. For example, different life stages and the different sexes are likely to differ in sensitivity to toxic stressors.

The extent to which endocrine disruptor effects are reversible also demands attention. The potential for effects being reversible may be much greater in invertebrates than in many vertebrates because of the remarkable regenerative abilities of some invertebrates. Evidence in support of this view is provided by the shore crab, *Carcinus maenas*. When the rhizocephalan barnacle, *Sacculina carcini*, parasitises the host male crab, it takes over hormonal control so that the male becomes castrated and takes on the characteristics of a mature female (Warner, 1977). However, when the parasite dies, the characteristics of the male re-emerge as it re-establishes hormonal control.

This review aims to (i) to summarize the key elements of invertebrate endocrine systems (Chapter 2); (ii) to assess whether existing test systems are adequate for the detection of endocrine disruption in invertebrates, what new tests might be required, which species of invertebrates are most appropriate for such tests, what end-points should be measured and whether the same organisms can be used for both laboratory and environmental monitoring (Chapter 3); (iii) to review the published evidence for endocrine disruption in aquatic invertebrates (marine and freshwater; experimental and field studies) resulting from exposure to chemicals in the environment (Chapter 4); (iv) to summarize the relevant UK and European legislation impacting on the monitoring and management of freshwater ecosystems (Chapter 5); (v) to discuss the information provided in the preceding Chapters, highlight the major gaps in knowledge and provide recommendations to improve our understanding of the processes underpinning endocrine disruption in invertebrates.

This review has been used to provide information for the SETAC-Europe and North America / OECD / EC Expert Workshop on Endocrine Disruption in Invertebrates: Endocrinology, Testing and Assessment (EDIETA) 12-15 December 1998 meeting and should be used in conjunction with the report from this meeting (Defur *et al*., 1999).

2. AN OVERVIEW OF THE ENDOCRINE SYSTEMS OF INVERTEBRATES

Our understanding of the nature and function of the endocrine systems of the major invertebrate phyla varies from extensive to superficial, and for those phyla which are best documented, data are often available for only a limited number of species.

A full and detailed review of the field of invertebrate endocrinology is beyond the scope of this report. Instead, a limited description is provided of the major components of the endocrine system in each major invertebrate group (where documented) together with citations of recent review articles (if available) and relevant research papers. The major phyla have been examined in descending order of research effort.

The majority of invertebrate hormones identified to date are peptide neurohormones. As yet there is no firm evidence for direct action of endocrine disrupting chemicals in peptide-based signalling systems. However, key insect and crustacean hormones are non-peptide in nature (ecdysteroids, juvenile hormones) and processes in which these hormones are involved may conceivably be more susceptible to receptor-related interference. In addition, the likelihood that disruption of endocrine processes may occur by interference at a locus other than the hormone receptor, such as a key biosynthetic enzyme, must be considered.

Summary of the invertebrate Phyla considered in this overview:

1. Phylum Arthropoda, class Insecta
2. Phylum Arthropoda, class Crustacea
3. Phylum Mollusca
4. Phylum Echinodermata
5. Others
    - 5.1 Phylum Coelenterata
    - 5.2 Porifera
    - 5.3 Acoelomata:
    - 5.4 Aschelminthes
    - 5.5 Annelida
    - 5.6 Protochordata

2.1 Insecta

The insects comprise 75% of all described species and occur in all habitats although very few species are truly marine. Insects are characterised by a tracheal respiratory system, a flexible exoskeleton or cuticle, and wings. Their life cycle is punctuated by a series of moults and juvenile forms (larvae) may differ in form and function from the adults (Kozloff, 1990). The field of insect endocrinology is extensive and the endocrine system of insects is the most-studied and best understood of all the invertebrate groups. The complexity of the insect endocrine system rivals that of the vertebrates. Consequently, only the most superficial account of the endocrine system of insects will be presented here, based on material collated primarily from review

articles and, to a lesser extent, from original research papers. Numerous reviews of various areas of insect endocrine function are available and the following summary was compiled from Highnam and Hill (1977), Bollenbacher and Bowen (1983), Akai (1987), Wigglesworth, (1972) and Cymborowski (1992) together with other more specific references provided within the text. As is the case for vertebrates, only a very limited number of insect species have been investigated fully. To avoid an unnecessarily complex presentation, the following account makes no attempt to identify species differences in function, rather, the endocrine system described is that of a Atypical≅ insect.

2.1.1 The insect endocrine system

In common with the endocrine systems of vertebrates, there is close coordination within the insect endocrine system between specialised nerve cells (neurosecretory cells) releasing neurohormones into the haemolymph (blood) and specialised endocrine glands synthesising and secreting hormones which are also released into the haemolymph. The major endocrine/neurohaemal organs of insects are the brain, the corpus allatum (a paired or fused structure, the corpora allata, CA), the corpus cardiacum (usually a paired structure, the corpora cardiaca, CC), the prothoracic glands, the frontal ganglion, the suboesophageal ganglion and the thoracic ganglia. The major functions and secretions of these tissues are described below.

Neurosecretory cells in the brain

Together with the CC the brain neurosecretory cells form the cerebral neurosecretory system. Neurosecretory products are synthesised by nerve cells and travel along the axon to the site of release. A major product of the brain neurosecretory cells is the peptide eclosion hormone (EH) which is transported to the CC for release.

The corpora cardiaca

These organs are located between the brain and corpora allata. The CC contain extrinsic cells, which store and secrete neurohormones produced in the brain or in ganglionic cell bodies, and intrinsic cells whose cell bodies are located within the CC and which synthesise and release their own hormones. More than fifty unique peptide structures have been described in the CC, responsible for the regulation of metabolism, pigment concentration, diuresis, moulting, and pheromone biosynthesis (Konopi│ska *et al*., 1992). Hormones released by the CC include adipokinetic hormone (AKH), prothoracotropic hormone (PTTH), eclosion hormone (EH), bursicon, hyperglycaemic hormone, melanization and reddish coloration hormone (MRCH, also known as pheromone biosynthesis and activating neuropeptide, PBAN)

AKH is a member of a large family of structurally related peptides (8-10 amino acids) which includes red pigment-concentrating hormone (RPCH), a chromatotrophic hormone found in Crustacea (Gäde, 1996). In functional terms, AKH is considered to act as a glucagon analogue, stimulating lipolysis in the fat body and inhibiting protein synthesis (Konopi│ska *et al*., 1992). PTTH, a peptide, is the initial component of the endocrine sequence leading to moult and its primary role is to stimulate release of ecdysone from the prothoracic glands (see below). It is believed to be a homodimeric protein with a high degree of species specificity. As little as 50% homology in amino acid sequence is reported to exist between species (Noguti *et* al., 1995; Suzuki *et* al., 1997). EH is responsible for initiating ecdysis-related behaviour and, together with the peptide bursicon, controls cuticle plasticization (Reynolds, 1985; Reynolds and Truman,

1983; Truman, 1985). Hyperglycaemic hormone (or hypertrehalosemic factor, a peptide; Goldsworthy and Gade, 1983) elevates trehalose levels in the haemolymph and promotes uptake of diacylglycerol into the fatbody

In addition, a follicle cell tropic hormone is produced by the CC; egg developmental neurosecretory hormone (EDNH) (Gersch, 1983)

The corpora allata

These paired or fused tissues show great structural variability between species. The CA secrete juvenile hormone (JH; homosesquiterpenoid epoxides, uniquely exploited in this context with only one other known example, ant trail pheromones). The CA is also a neurohaemal organ, secreting neurohormonal materials originating from the brain.

JH initially functions as metamorphosis modulator in larval insects, controlling growth and differentiation of insect epidermal cells, and then may function as a gonadotropin. There are a minimum of 5 congeners (JHI, II, III, 0, B_3) which co-exist and may exhibit different activities in bioassays but JHIII is now considered to be the predominant insect JH although this has yet to be confirmed for all major insect groups (Chang, 1993; de Kort and Granger, 1996).

JH secretion is in part controlled by feedback loops acting through the CNS and direct innervation of the CA (Tobe and Feyereisen, 1983) and JH is transported in association with haemolymph binding proteins (King, 1983). The ovary also influences the CA. Ovariectomy suppresses JH biosynthesis but the ovary also regulates the decline of JH associated with the end of the gonadotrophic cycle. JH regulates the synthesis of specific larval and adult proteins in the fat body (Laufer and Borst, 1983). These include vitellogenin and haemolymph haemoglobins.

JH is believed to act via an intracellular receptor in a manner similar to the mechanism employed by steroid hormones, regulating the rate of transcription of target genes but the receptor so far eludes identification (Jones, 1995; Riddiford, 1996).

The prothoracic glands

These are paired organs which, during postembryonic development, secrete ecdysone (an ecdysteroid, a member of a family of related steroids) primarily under the control of the neurohormone, PTTH.

Ecdysone is hydroxylated to 20-hydroxyecdysone (20-HE) in the fat body and malpighian tubules and enters the haemolymph where it may bind to proteins (Goodman, 1983). 20-HE is primarily inactivated by conjugation.

In adult insects the ovaries synthesise large quantities of ecdysteroids which are believed to have a variety of functions and are present in large amounts in eggs. During periods of embryonic cuticulogenesis large peaks of free ecdysteroids can be detected, possibly arising from the hydrolysis of maternal conjugates. (Hoffmann and Hetru, 1983).

Ecdysone and 20-hydroxyecdysone exhibit three major differences compared to vertebrate hormonal steroids. In ecdysone, the side chain of the precursor cholesterol has not undergone

cleavage; the AB ring junction in ecdysone is of the cis type and the A ring is almost perpendicular to the rest of the steroid nucleus; ecdysone and 20-hydroxyecdysone are hydrophilic as opposed to lipophilic like vertebrate steroids. Ecdysone operates, as do mammalian steroids, via intracellular receptors which mediate transcriptional activity (Koolman and Spindler,1983; Riddiford and Truman, 1993; Henrich and Brown, 1995). A review of the ecdysteroid biosynthetic pathway is provided by Grieneisen (1994).

2.1.2 Development and moulting

Insects, constrained within a rigid exoskeleton, exhibit discontinuous growth in which postembryonic development is punctuated by moult cycles, during which the old cuticle is shed (ecdysis) and replaced by a new one. The process of development and moulting is coordinated and controlled by elements of the endocrine system. Detailed reviews of the processes involved are provided by Tobe and Feyereisen (1983), Koolman and Spindler (1983), Laufer and Borst (1983), and Truman (1985), Riddiford (1996) and Riddiford and Truman (1993).

Various extrinsic and intrinsic factors, outside the scope of this summary, stimulate the release of PTTH from the CC into the haemolymph. PTTH stimulates the production of ecdysone by the prothoracic glands. The conversion of ecdysone to its active metabolite, 20-hydroxyecdysone, in the fat body signals the epithelial cells to begin processes leading to moult. At the same time, at all moults, except the final one, the CA secrete JH to ensure the development of another larval instar, rather than metamorphosis. The receptor which mediates the action of ecdysone is a member of the steroid receptor superfamily while the putative JH receptor is not a member of any known family of receptors (Riddiford, 1996). The final steps in the moult cycle are controlled by the hormones bursicon and eclosion hormone (EH). Eclosion hormone is secreted in response to declining haemolymph ecdysteroid levels and triggers a precisely coordinated behavioural sequence resulting in escape from the exuviae (Horodyski, 1996). In turn, declining EH levels stimulate the secretion of the neurohormone bursicon which facilitates cuticle plasticization and then tanning and hardening (Mulye, 1992).

2.1.3 Reproduction

The endocrine control of insect reproduction has been comprehensively reviewed by Davey (1983), Engelmann (1983), Hagedorn (1983, 1985), Girardie (1983), Huebner (1983), Koeppe *et al*. (1985), and Raabe (1986). The subject is complex and the summary presented here is of necessity much simplified.

Insect reproduction is primarily controlled by the CA. JH is the principal gonadotropin in many insect species, controlling vitellogenesis by the fatbody, influencing previtellogenic oocyte development and facilitating vitellogenin uptake by the oocytes. JH also promotes the maturation of the male accessory sex glands (see below) and is involved in controlling reproductive behaviour.

Oogenesis and spermatogenesis appear to be regulated by ecdysone, while both processes are inhibited by JH. Ecdysone may be produced locally in both the ovary and testes. Vitellogenesis in insects occurs largely in the fat body, although in some species it has been reported to take place in the ovary also (Valle, 1993). Vitellogenesis is controlled by JH and/or ecdysone. Corpora allata are essential for vitellogenesis in some species but not in others.

Factors with antigonadotropic activity are produced by mature oocytes, embryos or neurosecretory tissue associated with the ovary. The antigonadotropic factor(s) inhibit oocyte growth and may block the action of JH elsewhere. Neurosecretory hormone from the cerebral neurosecretory system is involved in ovulation, oviposition and parturition. Both ecdysone and JH may be involved indirectly in these processes also.

In some species the male deposits sperm in a spermatophore which is produced, together with additional secretions, by the male accessory glands. The secretory products of the accessory glands, incorporated into the spermatophore, may exert important effects in the female on oviduct contractions, oviposition, and oocyte production.

2.1.4 Diapause

Diapause is a term used to define the periodic interruption of development employed by insects to permit survival under adverse environmental conditions (Cymborowski, 1992). Diapause is observed to occur at all life stages of the insect, egg, larva and adult, and is hormonally regulated. Reviews of the hormonal control of diapause are provided by Yamashita (1983), Chippendale (1983), de Wilde (1983), Denlinger (1985), and Lavenseau *et al*. (1986).

In brief, environmental signals (day length, temperature) trigger a decision to enter diapause. The control of diapause appears to be diverse and may include the presence of a diapause hormone (DH), a requirement for JH, or the absence of JH, or a requirement for the absence of PTTH resulting in ecdysone deficiency.

2.1.5 Vertebrate-type steroids in insects

Estradiol-17β, estriol, testosterone, cortisol, progesterone and 17α-hydroxyprogesterone have been detected in whole-body homogenates and specific tissues of a wide range of insect species by gas chromatography-mass spectrometry (GC-MS), and by the less precise method of radioimmunoassay (RIA; Mechoulam *et* al., 1984; Ohnishi *et* al., 1985; De Loof and De Clerck, 1986; Denlinger *et al*., 1987; Lafont, 1991; Bradbrook *et al*., 1990) leading to speculation that these compounds have functional roles to play in the insect endocrine system. However, although there are reports of specific binding sites for steroids in insect tissues suggestive of the presence of receptors (Paesen and De Loof, 1988), other studies have failed to find evidence for nuclear binding of vertebrate-type steroids (Bidmon and Stumpf, 1991). Attempts to demonstrate a role for vertebrate-type steroids in insects have yet to prove successful (Ogiso and Ohnishi, 1986; Denlinger *et al*., 1987; Lafont, 1991) although variations in the concentration of pregnenolone, determined by RIA, which occur in normally developing *Sarcophaga bullata* larvae were absent in larvae treated with the JH analogue methoprene (Novak *et al*., 1990). Furthermore, Swevers *et al*. (1991a) evaluated the occurrence of synthetic pathways for vertebrate-type steroids in insects and concluded that there is no widespread evidence for the biosynthesis of vertebrate-type steroids from cholesterol and that vertebrate-type steroids Aprobably do not act as physiologically active substances in insects≅. In a review of the occurrence of ecdysteroids and vertebrate-type steroids among the invertebrates, including arthropods, Lafont (1991) also concluded that no firm evidence yet exists to indicate the role of vertebrate-type steroids which have been detected within insect tissues. It is widely conceded that the steroids may be present in the insects as a consequence of the ingestion of steroid-containing food.

2.2 Crustacea

There are almost 30,000 described species of Crustacea, the majority of which are marine, although there are numerous freshwater and some terrestrial groups also (Kozloff, 1990). The field of crustacean endocrinology is considerably less expansive than that of insect endocrinology. Fingerman (1987) observes that most of what is currently understood about the crustacean endocrine system has resulted from studies with decapods, although information on the amphipods and isopods is becoming more available. For the purposes of this report, the description of the crustacean endocrine system has been grossly simplified and is intended to represent a depiction of an idealised Atypical≅ crustacean, although no such animal exists.

2.2.1 The crustacean endocrine system

As is the case for insects, many behavioural and physiological processes in crustaceans are regulated by neurohormones. These include circadian and tidal rhythmicity, locomotion, posturing, chromatic adaptation, a variety of metabolic functions including glycogen and lipid metabolism, water and ionic balance, moulting, growth, regeneration, gonadal development, reproductive physiology, digestion and cardiac activity (Beltz, 1988; Taketomi *et* al.,1987). The summary presented here is a much-simplified account of current knowledge of the crustacean endocrine system and draws in particular on the reviews of Quackenbush (1986), Laufer and Downer (1988) and Fingerman (1987,1997a) together with other sources cited within the text. The major endocrine/neurohaemal organs of crustaceans are the X-organ sinus gland system, the postcommissural organs, the pericardial organs, the Y organ, the androgenic gland, the mandibular organs, and the ovaries. The major functions and secretions of these tissues are described below.

The sinus gland (SG)

This is located in the eyestalk. The sinus gland is a neurohaemal organ which stores and releases neurohormones produced elsewhere, predominantly in the X-organ. In crustaceans lacking eyestalks, the sinus glands lie within the head, adjacent to the brain.

The X-organ

This is a cluster of neuroendocrine cell bodies in the medulla terminalis, which provide most of the axons whose terminals compose the SG

The **X-organ SG system**, the major neuroendocrine regulatory centre in Crustacea, is structurally and functionally analogous to the vertebrate hypothalamo-neurohypophyseal system as well as to the corpus cardiacum of insects. A major product of the X-organ SG system is moult inhibiting hormone (MIH) a neuropeptide (72-78 amino acids) which exerts a negative control on ecdysteroid secretion by the Y organ (see below). This negative control of steroidogenesis which is observed in crustaceans is not typical of other groups, for example insects (Lachaise *et* al., 1993).

MIH is a member of a peptide hormone family which includes crustacean hyperglycaemic hormone (CHH) and vitellogenesis inhibiting hormone (VIH; gonad inhibiting hormone, GIH)

(Chang, 1993) which are also secreted by the SG. CHH appears to function under conditions of stress to rapidly elevate haemolymph glucose concentrations. The precise manner in which this hyperglycaemia is achieved, and the exact physiological function of CHH, are yet to be determined (Santos and Keller, 1993).

In addition to MIH, the X-organ SG system secretes a number of peptides concerned with pigmentary control in the integument, including red pigment-concentrating hormone (RPCH; or erythrophore concentrating hormone, ECH), white pigment-concentrating hormone (WPCH; or leukophore concentrating hormone, LCH), black pigment-dispersing hormone (BPDH; or melanophore dispersing hormone, MDH). The X-organ SG also releases hormones which affect the retinal pigments, light- and dark-adapting distal retinal pigment hormone (LAH, DAH) which act to adapt the compound eyes to differing levels of light intensity (Beltz, 1988).

Eyestalk also contains neurodepressing hormone (NDH) which depresses neuronal responsiveness. The SG system also secretes a substance which inhibits gastric mill activity.

The postcommissural organs
This is an additional neurohaemal organ, consisting of axon terminals, derived from the postcommissural nerves, that store and release neurohormones. Its function appears to be limited to the release of a number of chromatophorotropic peptide hormones

The pericardial organs
These are neurohaemal organs which release several different amine and peptide hormones, including cardioexciter peptides. Two cardiactive peptides have been characterised, proctolin a pentapeptide also found in insects and crustacean cardioactive peptide, a nonapeptide (CCAP; Fingerman, 1997a).

The Y-organ
This is a paired gland which is analogous to the prothoracic gland of insects and secretes ecdysone under inhibitory control by a moult-inhibiting hormone (MIH) from the X-organ sinus-gland complex (see above). Ecdysone is converted peripherally to 20-hydroxyecdysone which is the active form of the hormone. The situation in crustaceans is somewhat more complicated than in insects in that in certain species a combination of ecdysone and/or two other major secretory products, 25-deoxyecdysone and 3-dehydroecdysone, may be released by the Y-organ (Lachaise *et* al., 1993).

The androgenic gland
This is present only in males and is believed to be involved in the control of differentiation and function of the male reproductive tract. It performs the endocrine role normally attributed to the testes in vertebrates. The nature of the hormone(s) secreted by the androgenic gland have yet to be elucidated but initial evidence suggests they are proteinaceous. Current understanding of the role of the androgenic gland is summarised by Sagi *et* al. (1997).

The mandibular organs
These are situated close to the Y-organs. A distinct role has yet to be determined for the mandibular organs. However, they secrete methyl farnesoate (MF) a sesquiterpenoid and closely

related to insect JHIII. Production of MF by the mandibular organs is apparently under the control of the X-organ SG complex which secretes a mandibular organ-inhibiting hormone (MOIH) although other peptides may also be involved (Homola and Chang, 1997).It is suggested that MF is involved in the regulation of protein metabolism, the moult cycle and reproduction. The significance of the occurrence of JH-related compounds in both insects and crustaceans is considered by Cusson *et* al. (1991).

The ovaries

These are a source of one or more hormones which control the differentiation of female secondary sexual characters. The testis do not appear to be endocrinologically active.

2.2.2 Moulting

The majority of data regarding the endocrine control of moulting in crustaceans are derived from studies on decapod crustaceans (Chang and O'Connor,1988). The decapod (ten-legged) crustaceans include shrimps, lobsters, crayfishes, and crabs. A recent review of moulting processes in decapod crustaceans is provided by Chang (1995). Similar process have been well documented in other crustacean groups eg. Cirripeds.

The crustacean moulting hormone, crustecdysone, is 20-hydroxyecdysone, as in insects. Ecdysone is secreted by the Y-organ and rapidly hydroxylated by several tissues. As noted previously, it is now apparent that two other major secretory products, 25-deoxyecdysone and 3-dehydroecdysone, may be released by the Y-organ. Ecdysteroids are transported unbound in the haemolymph and exert their effects following binding to an intracellular receptor protein within the target tissue (Chang and O'Connor, 1988). The crustacean Y-organ is under the inhibitory control of moult-inhibiting hormone (MIH), a member of the CHH/MIH/VIH peptide family, secreted by the X-organ SG. It has been suggested that it is premature to designate the peptide known as MIH as the primary crustacean MIH until more extensive interspecific studies have been carried out (Chang *et* al., 1993). There is also evidence for a factor stimulating Y-organ activity in that methyl farnesoate, an unepoxidated precursor of insect JHIII produced by the crustacean mandibular organ, stimulates ecdysone production by the Y-organ (Laufer and Borst, 1988; Chang *et* al., 1993). A binding protein specific for MF has been detected in haemolymph of lobster (Chang *et* al., 1993). In addition, further inhibition of ecdysteroid synthesis by the Y-organs may be mediated by two newly-discovered compounds, 3-hydroxyl-L-kynurenine and xanthurenic acid (Fingerman, 1987).

2.2.3 Reproduction

Most attention has been focused on the reproductive endocrinology of amphipods (e.g. *Gammarus* sp.) and isopods (e.g. *Asellus* sp.). The subject of crustacean reproductive endocrinology has been reviewed by Fingerman (1987), Charniaux-Cotton and Payen (1988), and Hasegawa *et* al. (1993) among others.

The X-organ SG complex secretes a gonad-inhibiting hormone (GIH or vitellogenesis-inhibiting hormone, VIH) in both sexes and also a gonad-stimulating hormone (GSH or vitellogenesis-stimulating hormone, VSH). In females, GIH and GSH act on the ovary suppressing, or eliciting, the secretion of ovarian hormones and influencing vitellogenin synthesis by both ovarian tissue and the hepato-pancreas (Laufer *et* al., 1993). In the male the GSH and GIH act on the

androgenic gland. Whether or not ecdysteroids are necessary for the completion of vitellogenesis appears to be a species characteristic. GSH release from the brain is stimulated by 5-hydroxytryptamine (5-HT) and RPCH while dopamine and met-enkephalin indirectly inhibit gonadal maturation in both sexes (Fingerman, 1997b).

The androgenic gland, whose development is under genetic control, stimulates and coordinates the differentiation of the male reproductive system and development of male secondary sexual characteristics, including behaviour, via, it is inferred, the peptide androgenic gland hormone (AGH) and is under the control of the X-organ SG factors GSH and GIH. The testes are not endocrine organs, the androgenic gland performing that function instead (Sagi, 1988; Sagi *et* al., 1997).

In the female, the primordial androgenic gland does not develop and autodifferentiation of the ovaries occurs. A hormone secreted by primary follicular cells, permanent ovarian hormone (POH), controls the development of female characteristics. An additional hormone secreted during secondary vitellogenesis controls the formation of temporary external characters. This has been termed temporary ovarian hormone (TOH; Charniaux-Cotton and Payen, 1988). TOH may also be vitellogenin-stimulating ovarian hormone (VSOH) which promotes the synthesis of vitellogenin in the hepato-pancreas.

Methyl farnesoate (MF), produced by the mandibular organ, has also been implicated in the control of reproductive processes in crustaceans. Levels in the haemolymph vary according to the stage of reproductive development in both sexes (Laufer *et* al., 1993) and MF synthesis appears to be under inhibitory control by a factor secreted by the X-organ SG complex.

2.2.4 Metabolic Hormones in Crustacea

The SG releases crustacean hyperglycaemic hormone (CHH) (Keller and Sedleimer, 1988). However, the physiological role of CHH in metabolic processes is currently unclear (Santos and Keller, 1993). CHH receptors exist in the membranes of several tissues with cAMP and cGMP as second messengers. Activation of the system causes an inhibition of glycogen synthase and an activation of phosphorylase resulting in breakdown of glycogen and increased glucose levels in the hemolymph. CHH also stimulates amylase secretion by the hepatopancreas, the significance of which has yet to be defined.

2.2.5 Control of Water Balance

Seawater is isosmotic with the haemolymph of most crustaceans although in some cases the environment is hyperosmotic. In freshwater, the medium is hypoosmotic. Data are only available for decapod crustaceans and these are limited and suggest considerable variability in the mechanisms of control of water balance (Muramoto, 1988). There is, however, strong evidence that the neurohormonal centres produce substances which are involved in ionoregulatory processes. Factors both inhibiting and promoting water influx have been reported (Fingerman, 1997a).

2.2.6 Pigment Hormones

Crustaceans have two types of pigmentary effectors - chromatophores and retinal pigments (Fingerman, 1988). The combination of, for example, black, red, yellow and white

chromatophores allows complex integumental colour change to be achieved. Dispersion and aggregation of the pigment granules within epidermal and retinal cells are controlled by pigmentary effector neurohormones, all of which are peptides, released from the X-organ SG complex and postcommissural organ. Hormones so far identified include red pigment-concentrating hormone (RPCH; or erythrophore concentrating hormone, ECH), white pigment-concentrating hormone (WPCH; or leukophore concentrating hormone, LCH), black pigment-dispersing hormone (BPDH; or melanophore dispersing hormone, MDH).

The X-organ SG also releases hormones which affect the retinal pigments, light- and dark-adapting distal retinal pigment hormone (LAH, DAH) which act to adapt the compound eyes to differing levels of light intensity (Beltz, 1988).

The pigment-concentrating hormones of Crustacea show structural homology with insect adipokinetic hormones (AKH) secreted by the corpora cardiaca.

Release of the chromatophorotropic hormones from their storage and release sites is under the control of a number of neurotransmitters/neuromodulators including 5-HT, dopamine, norepinephrine, acetylcholine, histamine, octopamine, GABA, met-enkephalin. The role of biogenic amines in the control of pigmentation, and other functions, is reviewed by Fingerman *et* al. (1994).

2.2.7 Pheromones

There is a strong weight of evidence for the existence of crustacean pheromones. The subject is reviewed thoroughly by Bauchau (1986) and Dunham (1988). Although it is a subject that has received little consideration, pheromonal communication is likely to be susceptible to interference by compounds capable of mimicking pheromones.

2.2.8 Vertebrate-type steroids in crustacea

In a short review article de Loof and de Clerck (1986) discuss the early reports of vertebrate-type steroids in arthropods and conclude that C_{21} (progestogens, corticosteroids), C_{19} (androgens) and C_{18} (estrogens) steroids are widespread among the arthropod groups. However, despite the assertion by these authors that the endocrine systems of vertebrates and invertebrates have much in common, firm evidence for a role of vertebrate-type steroids in the crustacean endocrine system is still lacking, as is the case for insects. Three approaches have been employed to examine the role of vertebrate-like steroids in crustaceans; the ability of crustacean tissues to metabolise steroid substrates and analysis of the products produced by such metabolism provides information on the nature of the steroid metabolising enzymes present. The presence of vertebrate-type steroids has been sought directly by analyzing haemolymph and tissue extracts and the effects on processes such as vitellogenesis and moulting of administering vertebrate-like steroids to crustaceans has been evaluated.

In a study in which the metabolism of vertebrate-type steroids (pregnenolone, progesterone, 17α-hydroxyprogesterone and androstenedione) was examined in the tissues of three crustacean species (Swevers *et* al., 1991b) no evidence was found to substantiate claims that vertebrate-type steroids are involved in key aspects of crustacean function. Crustacean tissues failed to metabolise steroid substrates in a manner analogous to that of vertebrates and there was no

evidence for the active synthesis of vertebrate-type steroids. A similar conclusion was reached by Young *et* al. (1992) who investigated in detail the metabolism of progesterone by the hepatopancreas, gill, muscle and ovarian tissues of the decapod *Penaeus monodon*. The authors could detect no evidence for the presence of the enzymes known to be necessary to convert progesterone to androgens and estrogens in vertebrates.

Several reports have demonstrated the existence of biotransformation enzymes in *Daphnia magna* for which testosterone is a suitable substrate (Baldwin and LeBlanc, 1994a and b; Parks and LeBlanc, 1996). They demonstrated that the rate of elimination of the metabolites of testosterone is significantly reduced in *Daphnia magna* following exposure to DES (Baldwin *et al*, 1995), phenobarbitol modulates P450 activity in daphniids (Baldwin and Le Blanc, 1994) and some pesticides cause de-masculinisation in aquatic invertebrates (Le Blanc *et al*., 1997). However, the existence of a metabolic capability does not confirm an endogenous functional role for testosterone. The biotransforming enzymes may be of broad specificity and/or the organism may require a capability to metabolise and excrete steroids ingested with food items.

Attempts to directly identify vertebrate-like steroids in crustacean tissues have been successful for some species. Progesterone-like and estradiol-like immunoreactivity has been detected in lobster tissues (Couch *et* al., 1987), estradiol being present only in those animals with maturing ovaries. These authors suggested that estradiol may have a role in vitellogenesis in crustaceans. A marked association between vitellogenesis and estradiol levels was also observed in the anostracan *Artemia* in which progesterone, pregnenolone, 5α-dihydrotestosterone, testosterone, estrone and estradiol were detected by radioimmunoassay (Van Beek and De Loof, 1988). Changes in haemolymph vitellogenin and ecdysteroid levels were documented by Okumura *et al*. (1992) during reproductive and non-reproductive moult cycles in the fresh water prawn *Macrobrachium nipposense.* Variation in the levels of progesterone- and 17β-estradiol-like immunoreactivity in the haemolymph of the shrimp, *Pandalus kessleri,* was found to be related to the stage of vitellogenesis (Quinitio *et* al., 1991). These studies, and other similar reports, are open to criticism on the grounds that positive identification of the steroids causing displacement of the labelled ligand in radioimmunoassay is not possible. Lack of specificity in the antibody employed can lead to a number of structurally related compounds being effective in causing displacement.

A number of studies have been carried out in which more rigorous means of identifying the steroids present in crustacean tissues have been employed. In some cases, the results of these studies have supported the observations made with less rigorous methodology. Gas chromatography/mass spectrometry (GC/MS) with selected ion monitoring (SIM) was employed to examine the steroid content of tissue extracts from the decapod crustacean *Nephrops norvegicus* (Fairs *et* al., 1989). These authors confirmed the presence of 17β-estradiol in the eggs and haemolymph, and found 5α-dihydrotestosterone, testosterone and pregnenolone in ovarian tissue. Pregnenolone and estradiol-17β have also been identified in the tissues of the brine shrimp *Artemia* sp. by GC-MS (Novak *et* al., 1990). Similar results were reported for *Penaeus monodon* (Fairs *et* al., 1990) in which GC/MS with SIM revealed both 17β-estradiol and estrone to be present in the ovarian tissue during vitellogenesis. Testosterone was also detected in mature ovarian tissue. Using HPLC and RIA methods, progesterone-like and estradiol-like substances

were detected in ovaries, hepatopancreas and body fluids of the soldier crab, *Mictyris brevidactylus* (Shih, 1997). In contrast, HPLC and RIA failed to detect 17β-estradiol in haemolymph or tissue samples from blue crab, *Callinectes sapidus* (Sasser and Singhas, 1992), although estriol was detected. Androgens, estrogens and cortisol have been detected by HPLC in the haemolymph of *Penaeus japonicus*, some in both conjugated and unconjugated forms (Summavielle *et* al., 1995).

Although confirmation of a functional role for androgens, estrogens, or progestogens in crustacean endocrinology has yet to be provided, some newer data are suggestive of such effects. Induction of mitochondrial and cytosolic enzymes has been demonstrated to occur in a dose-dependent manner in the hepatopancreas of the prawn, *Macrobrachium rosenbergii*, following administration of 17β-estradiol (Ghosh and Ray, 1993a and b) and these authors have also reported that 17β-estradiol causes an accumulation of protein in the hepatopancreas, muscle and hemolymph of *M. rosenbergii* (Ghosh and Ray, 1992) and induction of lipogenic enzymes (Ghosh and Ray, 1994). Levels of estradiol-17β and progesterone vary significantly during vitellogenesis in the shrimp *Penaeus monodon* with peak levels of progesterone correlating with levels of vitellogenin (Quinitio *et* al., 1994). In contrast to the positive results of these studies, Koskela *et* al. (1992) failed to observe any effects of exogenous 17β-estradiol or 17α-hydroxyprogesterone on moult cycle duration or ovarian development in the tiger prawn, *Penaeus esculentus.*

In a review of the occurrence of ecdysteroids and vertebrate-type steroids among the invertebrates, including arthropods, Lafont (1991) concludes that, as is the case for insects, no firm evidence yet exists to indicate the role of vertebrate-type steroids within crustacean tissues. A similar conclusion is reached by Fingerman *et* al. (1993).

2.3 Mollusca

The molluscs are second only to the arthropods in terms of number of species, with more than 100,000 extant species. Hormones are involved in the control of reproduction, growth, energy metabolism, blood circulation and water and ionic metabolism in molluscs. However, little is known about the endocrine system of molluscan groups other than the Gastropoda (snails and slugs: subclasses Prosobranchia, Opisthobranchia and Pulmonata) and Cephalopoda (octopuses, squids). In particular, there is little published information concerning the endocrinology of bivalve molluscs (clams, oysters, mussels), despite the commercial importance of this group (Toullec *et* al., 1992).

2.3.1 The molluscan endocrine system

Published information is fragmentary and it is therefore difficult to present a coherent picture of a generalised molluscan endocrine system, particularly in view of the wide range of reproductive mechanisms present in the Gastropoda. The summary presented below is drawn primarily from the reviews of Joosse and Geraerts (1983), Joosse (1988) and Takeda (1987) and has been simplified. Because of the absence of a generalised Amodel≅ mollusc, the endocrine systems of the best-described groups will be considered in turn on a functional basis.

2.3.1a Prosobranch snails

This group represents the most primitive and diverse group of gastropods and shows wide variation in form and habitat. The majority are gonochoristic (Fretter, 1984). Little is known of the endocrine control of reproduction in this group. More attention has ben directed at the process of sex reversal and its endocrine control. Protandric sex reversal (a male phase precedes a female phase and these are separated by a hermaphrodite phase) occurs in certain species.

Masculinizing and feminizing factors have been identified/detected in the haemolymph which are neuroendocrine in nature. Sex reversal is believed to occur following the release of a feminizing factor by the brain. Regression of the male accessory sex organs (sperm duct, seminal vesicles and associated structures) is dependent on a neurohormonal factor, as is the dedifferentiation of the penis. The release of the factors responsible overall for modification of sexual status are controlled by social conditions.

Prostaglandins E or F have been shown to induce spawning in some marine snail species and have similar effects in other molluscs. A neurohormone is responsible for release of the gametes, egg capsule-laying substance (ECLS).

2.3.1b Opisthobranchia

These gastropods are exclusively marine and the majority are functional simultaneous hermaphrodites (Hagedorn *et al*., 1984). Endocrine-related research has focused mainly on egg-laying hormones of aplysiid species, which is reviewed thoroughly by Geraerts *et* al. (1991). Egg laying in the sea hare *Aplysia* is controlled by peptide products of the bag cells (BC), located at the rostral margin of the abdominal ganglion, which include egg-laying hormone (ELH), α-bag cell peptide and calfluxin (Geraerts *et* al.,1991).

2.3.1c Pulmonates

This group is comprised of two orders, freshwater snails (Basommatophora) and terrestrial snails (Stylommatophora). All are hermaphrodites (Tompa, 1984; Geraerts and Joosse, 1984) but reproduction involves mating and exchange of sperm. Among the gastropods, the endocrine system of the pulmonates is the best documented. A number of endocrine centres have been identified.

The dorsal bodies

These organs are present in all freshwater and terrestrial pulmonate gastropods and are named for their dorsal position on the cerebral ganglia. Paired mediodorsal bodies (MDB) may be accompanied by laterodorsal bodies (LDB) in some species. Structures similar to the DB have been reported for other groups. The tissue produces dorsal body hormone (DBH) and may be a site of synthesis of steroids. Ecdysone has been detected in the DB of at least two species of snail. DBH is a female gonadotrophic hormone which stimulates oocyte growth (vitellogenesis) and final oocyte maturation and also controls/maintains the female accessory sex organs (ASO).

The optic tentacles

These appear to produce a masculinizing substance which is required for the differentiation of male gonads, while females autodifferentiate. A masculinizing factor is also produced by the cerebral ganglion.

The caudo-dorsal cells
These are located in the caudodorsal part of the cerebral ganglia and produce peptides involved in induction and control of ovulation, egg mass formation and egg laying behaviour in freshwater pulmonates. Caudo-dorsal cell hormone (CDCH) is the best documented example. The peptides of this gland are released in large quantities only associated with egg-laying. CDCH as some influence on the female ASO

The lateral lobes
These elements of the cerebral ganglia produce secretory products which are involved in control of body growth and reproductive activity. The factor has an inhibitory action on the neurosecretory light green cells (LGC) which produce a growth hormone. The cerebral ganglia also produce a hyperglycaemic factor which inhibits glycogen synthesis and stimulates glycogen breakdown. An insulin-like factor is produced by the intestinal wall.

The gonad
In some groups, the gonad acts as an endocrine organ and steroid synthesising capacity has been demonstrated (see below). In freshwater pulmonates the female ASO are controlled by DBH. Control of male ASO is not documented. In terrestrial pulmonates the gonad does exert influence on the ASO, in addition to DBH.

The light green cell system (LGC; after staining with alcian blue**)**
These neurosecretory cells are located within the cerebral ganglia and release neurohormones which stimulate growth, including shell growth, protein and carbohydrate metabolism and calcium and sodium regulation. At least four insulin-related peptides are produced, molluscan insulin-related peptides (MIPs), which are related to other members of the insulin superfamily (Geraerts *et* al., 1991; Smit *et* al., 1991).

The dark green cell system (DGC; after staining with alcian blue**)**
This group of neurosecretory cells is located primarily within the pleural ganglia and produces a factor with diuretic effect. This hormone appears structurally analogous with vertebrate thyroid stimulating hormone-releasing hormone (TRH). A factor with natriuretic effects is secreted by the cerebral ganglia.

The yellow cells (YC) and yellow green cells (YGC)
These cell types react to different osmotic environments in a similar manner to the DGC. They produce sodium influx stimulating peptide (SIS).

2.3.1d Cephalopods
All cephalopods (*Nautilus*, squids and octopods) are gonochoristic. The optic gland is under inhibitory nervous control (by an FMRFamide-like peptide) and stimulates gonadal development (multiplication of oogonia and spermatogonia). Optic gland hormone stimulates vitellogenesis and growth and development of male and female ASO.

2.3.2 Vertebrate-type steroids in molluscs
Progestogens, androgens and estrogens have been reported to occur in at least three classes of

molluscs; the cephalopods, pelecypods (bivalves), and gastropods (Hines *et* al., 1996). These authors suggest that the published literature is strongly indicative of a widespread distribution of vertebrate-type steroids among molluscan species. Despite this, specific functional roles for many of the steroids detected in molluscs have yet to be attributed. The papers cited below do not represent an exhaustive assemblage of those published which address this subject but are intended to provide an entry into the literature. Many related papers, describing the occurrence of vertebrate-type steroids in molluscs, are to be found in the reference lists of those cited below.

As is the case for similar work on crustaceans and insects, a variety of methods have been employed to detect and quantify steroids in molluscan tissues. Gas chromatography - mass spectrometry (GC-MS) and radioimmunoassay (RIA) were employed by Reis-Henriques *et al*. (1990) to demonstrate the presence of progesterone, androstenedione, testosterone, 5α-dihydrotestosterone, estradiol-17β and estrone in *Mytilus edulis*. No marked sex-specificity was observed for these steroids. It was subsequently shown that whole-animal progesterone levels in *Mytilus edulis* displayed a distinct annual cycle in which peak levels were coincident with the main spawning season (Reis-Henriques and Coimbra, 1990). The authors suggest that the similar levels of progesterone found in males and females indicate that progesterone may be a precursor to other, more sex-specific, steroids. A similar approach has been used to identify steroids and relate their levels to physiological cycles.

The ability of *Helix aspersa* to metabolise radiolabelled androstenedione was studied by Le Guellec *et* al. (1987). It was found that androstenedione was converted to testosterone, 5α-dihydrotestosterone, androsterone and estriol suggesting that among other enzyme systems aromatase must be present. Direct analysis of steroids in the haemolymph by GC-MS revealed the presence of androsterone, dehydrepiandrosterone, androstenedione, 3α-androstanediol, estrone, estradiol-17β and estriol. Measurement of the levels of certain of these steroids in the haemolymph revealed that circulating concentrations were linked to reproductive activity. The authors concluded this to be evidence for a physiological role for endogenous steroids in reproductive processes.

A similar result was obtained more recently. Radioimmunoassay and *in vitro* biosynthesis from endogenous precursors was employed to examine the steroids present in *Achatina fulica* (the giant African land snail; Bose *et* al., 1997), progesterone, androstenedione, testosterone, estradiol-17β, and cortisol were detected in the haemolymph by RIA. Functional significance was attributed to the absence of estradiol-17β from male phase haemolymph. The ovotestis and albumen gland were found to synthesise progesterone, androstenedione, testosterone, and estradiol-17β. An earlier study examined the steroidogenic activity of the ovotestis and digestive gland of the snail *Lymnaea stagnalis*, using radiolabelled pregnenolone and subsequently determining the presence of progesterone in tissue homogenates by thin layer chromatography and recrystallisation of products to a constant specific activity (De Jong-Brink *et al*., 1981). The ovotestis were also found to be biosynthetically active in this species.

More specific evidence for a functional role for steroids in molluscan physiology has been provided by a number of studies. Henry and Boucaud-Camou (1994) demonstrated the ability of progesterone to promote the incorporation of radiolabelled glucose into the polysaccharides of

cuttlefish (*Sepia officinalis*) nidamental cells *in vitro*. The nidamental glands are responsible for providing a component of the capsule surrounding the mature oocyte and progesterone levels in the gonad of *Sepia* increase as sexual maturity advances. Recent support for a role for steroids in cephalopods is provided by a report that progesterone, testosterone and estradiol-17β, together with their respective high-affinity binding proteins, have been detected in the reproductive system of *Octopus vulgaris* (D'Aniello *et al*., 1996). In the Japanese scallop, *Mizuhopecten yessoensis*, estradiol, progesterone and testosterone have been shown to have a stimulatory effect on both oogenesis and development of the testes (Varaksina and Varaksin, 1991; Varaksina *et al*., 1992). Estradiol-17β also enhances the effects of serotonin on the release of eggs from the ovary (Osada *et al*., 1992) and has been implicated in the control of catecholamine levels in the gonad in the scallop *Patinopecten yessoensis* (Osada and Nomura, 1989).

The arthropod steroid ecdysone has also been detected in molluscs. The mediodorsal bodies (MDB) of the snails *Lymnaea stagnalis* and *Helix pomatia* and the ovotestis of *Helix* are reported to contain ecdysteroids, identified by HPLC and RIA (Nolte *et al*., 1986). Other reports of the existence of molluscan ecdysteroids also exist (Romer, 1979; Whitehead and Sellheyer, 1982; Garcia *et al*., 1986). However, in vivo and in vitro studies utilising radiolabelled precursors have failed to provide evidence for the synthesis of ecdysone in a number of gastropod snails. The authors concluded that if ecdysone was being synthesised endogenously the snails must be employing a biosynthetic pathway different to that of arthropods (Garcia *et al*., 1995).

2.4 Echinodermata

The Echinodermata represent a unique phylum, of about 6000 living species, with no clear relationship with any other phylum. They are radially symmetrical with a calcareous internal skeleton and a water vascular system. The best known classes comprise the starfish (Asteroidea), brittle-stars (Ophiuroidea), sea urchins (Echinoidea) and sea-cucumbers (Holothuroidea). Echinoderms do not possess a well-developed glandular endocrine system but complex chemically mediated interactions do occur between cells (Shirai and Walker, 1988; Shirai, 1987; Highnam and Hill, 1977). The hormonal control of spawning and maturation in the starfish has received a lot of attention and there is evidence that spawning in sea urchins may also be hormonally controlled. A most notable contrast with the other invertebrate groups discussed in this review is the strong evidence that vertebrate-type steroids play an important role in control and coordination of a number of functions in echinoderms. This overview draws heavily on the reviews of Shirai and Walker (1988), Cobb (1988) and Smiley (1990).

2.4.1 Hormone function in echinoderms

In echinoderms, asexual reproduction, which involves the autotomy of body parts and regeneration of lost structures, appears to require unique neurochemical factors. A peptide, gonad-stimulating substance (GSS, or radial nerve factor, RNF), is implicated in the control of sexual reproduction and gametogenesis. Known hormonal peptides do not mimic the action of GSS, which is localised in the radial nerves. Reinitiation of meiosis in primary oocytes involves GSS, maturation inducing substance (MIS) and maturation-promoting factor (MPF). GSS appears to act by stimulating follicle cells to produce MIS. MIS (1-methyladenine, 1-MeAde) is a purine base derived from 1-methyladenosine in the follicle cells, which surround the oocyte.

When oocytes are treated with 1MeAde, MPF is generated in the cytoplasm via an increase in cytoplasmic cAMP. MPF causes germinal vesicle breakdown and subsequent completion of maturation including breakdown of the follicular envelope. Both GSS and MIS are involved in stimulating discharge of the gametes, in both sexes. It is believed that control and coordination of vitellogenesis in echinoderms is mediated by steroids. In some species, nutrients utilised in vitellogenesis are initially stored and mobilised from the cells of the pyloric caeca and the processes involved in vitellogenesis in sea stars are reviewed by Voogt *et* al. (1985). Vitellin has been determined in sea urchin coelomocytes (Cervello, 1994) indicating that it may be variable, or multiple sites of producation may be present. The molluscan neuropeptide FMRFamide has also been reported occur in the nervous system of *Asterias rubens* (Elphick *et* al., 1989).

There has been a considerable amount of work published in recent years which focuses on the presence and role of steroids in echinoderms, much of it subsequent to the review of Shirai and Walker (1988). A representative selection of these papers will be discussed below.

2.4.2 Vertebrate-type steroids in echinoderms

Numerous studies have presented strong evidence that vertebrate-type steroids are present in echinoderms. Several of these have demonstrated a metabolic/synthetic capacity within echinoderm tissues for steroids. In a study employing cell-free homogenates of ovarian and pyloric caeca tissue of the starfish *Asterias rubens*, transformation of radiolabelled androstenedione to a number of metabolites, including testosterone, was demonstrated (Schoenmakers and Voogt, 1981), a result subsequently confirmed using intact tissue pieces rather than homogenates (Voogt and van Rheenen, 1986). A similar, more recent, report demonstrates the conversion of radiolabelled androstenedione to a number of androgen metabolites (including testosterone) by homogenates of body wall, testis and ovary from *A. rubens*. This indicated the involvement of endogenous androgens in the control of growth and reproductive processes (Hines *et* al., 1992a). This conclusion is supported by the results of Voogt *et al*. (1991) who showed that the conversion of dehydroepiandrosterone, progesterone and androstenedione in gonad and pyloric caeca of male and female *A. Rubens* was sex- and tissue-specific, also the degree of metabolism was related to the reproductive cycle. The biosynthesis of novel fatty-acyl steroids, fatty-acyl pregnenolone, mono-fatty-acyl androstenediol and fatty-acyl testosterone has been reported to occur in the sea star *A. rubens* (Voogt and van Rheenen, 1986; Voogt *et* al., 1990).

Similar congruence between the reproductive cycle and steroid metabolism has been reported for levels of estrogens and progesterone determined by RIA in the gonads and pyloric caeca of the asteroid *Sclerastias mollis.* Sex-dependent differences were apparent in the relative concentrations of the two steroids being detected in the gonad but not pyloric caeca (Xu, 1991). In *A. vulgaris* increases in levels of estradiol, progesterone, and testosterone coincided with events during gametogenesis (Hines *et* al., 1992b). Other studies have reported levels of progesterone and estrogen in reproductive tissues of sea stars (Schoenmakers and Dieleman, 1981; Voogt and Dieleman, 1984; Xu and Barker, 1990). However, despite the direct measurement of estrogens, there has been doubt regarding the capacity of echinoderm tissues to synthesise estradiol-17β or estrone. The identity of at least one estrogen measured by RIA has been confirmed as estradiol-17β by GC-MS (Voogt *et* al., 1992). In support of the functional role of estradiol-17β in echinoderm reproduction, receptor-like binding of estradiol-17β has

been reported in the pyloric caeca of *A. Rubens* (de Waal *et al*., 1982).

Evidence for the functional significance of steroids in echinoderms is accumulating. An early report describes that estradiol-17β promotes the growth of oocytes in cultured ovarian fragments of the starfish, *Asterina pectinefera* (Takahashi and Kanatani, 1981) and a similar effect was observed in *Asterias rubens* receiving daily injections of estradiol-17β (Schoenmakers *et* al., 1981). Injection of estradiol-17β or estrone into the starfish *S. mollis* caused an increase in estrone and progesterone levels in the ovaries, an increase in oocyte diameter, and higher protein levels. Increases in progesterone levels in the pyloric caeca were also observed (Barker and Xu, 1993). These authors speculate that these results suggest estrogens and progesterone are involved in the regulation of metabolic and reproductive processes. Changes in the levels of certain components of biosynthetic pathways in the asteroid *Luidia clathrata* were observed following estradiol-17β and estrone injections (Watts and Lawrence, 1987). Estradiol-17β also induced the synthesis of a novel protein, although not yolk protein, in the coelomycetes of an asteroid and two echinoids (Harrington and Ozaki, 1986).

2.5 Other groups

2.5.1 Coelenterata (Cnidaria)

In the less highly organised invertebrates, such as the coelenterates (comprising the jellyfish, sea anemones, corals and hydroids) which possess the simplest nervous system within the animal kingdom, epithelial endocrine glands are absent and neurosecretions are the primary hormonal coordinators (Highnam and Hill, 1977; Lesh-Laurie, 1988). There is very little published work on the presence and function of hormonal substances in this group.

The freshwater hydrozoan, Hydra, has been best investigated of this group of animals. Studies of growth, regeneration, and development of sexuality in species of Hydra have provided convincing evidence for the existence of peptide hormones in coelenterates, and their involvement in critical processes. For references, see Lesh-Laurie (1988). A specific review of the occurrence and function of Phe-Met-Arg-Phe-amide (FMRFamide) in Coelenterates is provided by Grimmelikhuijzen *et al*., 1988).

Vertebrate-type steroids and steroid-metabolizing enzymes have been detected in corals. Slattery *et al*. (1997) report the presence of enzymes capable of metabolising radiolabelled progesterone and androstenedione in the Antarctic soft corals *Alcyonium paessleri* and *Clavularia frankliniana* and demonstrated the presence of progesterone, androstenedione, testosterone and estradiol-17β in tissues of these species using RIA. These authors also summarise the evidence for steroid occurrence among corals, including the release of estradiol-17β during mass spawning of corals (Atkinson and Atkinson, 1992), leading to speculation that estradiol-17β may have a functional role in regulating reproductive processes in this group of invertebrates.

2.5.2 Porifera

The presence of a nervous system in members of the Porifera (sponges) is uncertain, although cells with the appearance of neurones have been described (Highnam and Hill, 1977). However, representatives of this phylum are generally considered not to possess a nervous system,

digestive tract, or specialised structures for respiration and excretion. Most sponges are hermaphrodite but produce eggs and sperm asynchronously. Asexual reproduction, by budding occurs in some species (Kozloff, 1990). We have been unable to identify any further sources of information on the presence or otherwise of hormone function in this group.

2.5.3 Acoelomata (Phylum Platyhelminthes, Phylum Nemertea)

This group comprises the flatworms (Turbellaria, Digenea, Monogenea, and Cestoda), planarians, flukes, tape-worms, and ribbon worms. Platyhelminthes do not possess true endocrine glands or a circulatory system. Products released from neurosecretory centres reach target tissues by diffusion throughout the extracellular spaces or by direct delivery to the vicinity of target cells. In the Nemerteans, the ribbon worms, hormones are probably released into the blood space (Webb, 1988).

According to Webb (1988) neurosecretory cells have been identified in the cerebral ganglia of several species of platyhelminthes and neurosecretory products appear to be involved in regeneration and reproduction. Involvement of neurohormonal factors in aspects of asexual reproduction is also postulated. During sexual reproduction a neurosecretion stimulates the maturation of the ovaries and testes, which in turn stimulates the formation of the copulatory apparatus. The factor produced by the testis is presumed to be a steroid (possibly testosterone) and inhibits division while providing a negative feedback signal to the testes. Immunoreactivity to various vertebrate neurohormones, including somatostatin, neurophysins, ACTH, and met-enkephalin, has been detected. The nature of the reacting sites is not known. In some species vertebrate MSH-release-inhibiting factor (MIF) causes contraction of the chromatophores, possibly by acting on a pigment dispersing hormone from the brain. Serotonin and tryptamine may also have effects. In the Nemertea, neurosecretory products may be involved in osmoregulation and gonadal development is under suppressive control by the cerebral ganglia and related organs via a Gonad inhibiting factor.

Thorough reviews of progress in this field, concentrating on regulatory neuropeptides in parasitic platyhelminths, are provided by Halton *et* al. (1990) and, Fairweather and Halton (1991, 1992). Reuter and Gustafsson (1996) summarise what is known of the role of neuropeptides in control of multiplication and development in all the major flatworm taxa. Fairweather and Skuce (1995) in their review of neuropeptides in flatworms emphasise that although progress has been made in identifying and chemically characterising platyhelminth neuropeptides, a full understanding of their functional significance is still lacking.

We have been unable to find any systematic evidence in the literature that steroids have a functional role in regulatory processes in the acoelomates.

2.5.4 Aschelminthes

This group includes the Phylum Rotifera, Phylum Gastrotricha and Phylum Nematoda and is characterised by the first appearance of a fluid filled body cavity. Other than nematodes (round worms) little is known of the endocrinology of members of this phylum. The parasitic nematodes (e.g. *Ascaris*, the intestinal parasite of mammals) are not considered relevant to the scope of this review. The endocrinology of the nematodes has been reviewed by Davey (1988).

Davey (1988) emphasises that no functions in the Aschelminthes have been shown unequivocally to be controlled by hormones, allthough growth, cuticle formation, ecdysis and gonad development are among the postembryonic developmental events in nematodes that, by analogy with other animals, could be hormonally controlled. Neurosecretory cells (peptidergic) have been described histologically by light and electron microscopy. Neurosecretory cells (aminergic) - noradrenaline, dopamine, 5-HT, octopamine have all been identified in nematode extracts. The biochemical machinery for the synthesis of catecholamines has also been identified. Despite the reports of immunoreactivities to a variety of peptides in the nervous system of nematodes (Brownlee *et al*., 1993), all the peptides isolated to date and considered to be of endogenous origin are FMRFamide-related peptides (Brownlee *et al*., 1996).

Nematode life cycle is punctuated by moulting events. Therefore the question of the existence of a moulting hormone (JH) has arisen. JH controls both moulting and metamorphosis in insects. All JH variants, which have been identified, are derivatives of the methyl ester of 10,11 epoxy-farnesoic acid. There is limited evidence for the occurrence of these compounds in nematodes. JH and its analogues can cause the release of noradrenaline from neurosecretory cells in the cephalic papillary ganglion. Noradrenaline, acting as a neurotransmitter/ modulator/hormone, acts to cause release of an ecdysial hormone which in turn causes the secretion of enzymes into the space between new and old cuticle. Both ecdysterone and 20-OH-ecdysterone have been definitively identified in some nematodes although the biosynthesis of ecdysteroids by nematodes has yet to be confirmed (Barker and Rees, 1990; Barker *et al*., 1990). Although JH and its analogues have effects on nematodes these are at concentrations well in excess of those required for effects in insects. Similar conclusions have been drawn regarding ecdysone.

2.5.5 Annelida

The annelids (the segmented worms) are considered to be closely allied to the phylum Arthropoda. They are the most structurally complex of the worms, possessing a complete gut and coelom with a well-developed circulatory system in many species. The phylum is divided into three major subdivisions: the class Polychaeta (bristle worms, e.g. *Nereis*), class Oligochaeta (e.g. earthworm, *Lumbricus*) and class Hirudinea (the leeches). Neurosecretory cells have been described in the brains or supra-oesophageal ganglion of polychaetes, oligochaetes and hirudinea and the general endocrinology of these groups is reviewed by Highnam and Hill (1977) and Takeuchi (1987). The identity of neuropeptides detected in annelids is summarised by Porchet and Dhainaut-Courtois (1988) and an overview of the immunocytochemistry, function, and sites of synthesis of neuropeptides in annelids is provided by Al-Said and Al-Yousuf (1992).

In a recent review, Bentley and Pacey (1992) summarise the endocrine involvement in reproductive processes in polychaete worms and make the general point that although numerous polychaete hormones are assumed by inference to exist, in very few cases is the chemical identity of the hormones established. The cerebrovascular complex (CVC) is a neurohaemal organ in the ventral region of the brain within which exists an infracerebral gland. Secretory end feet (SEF) are characteristic axonal endings of neurosecretory cells. A sexual maturation inhibitory hormone (juvenile hormone), a gonadotrophic hormone, a sperm maturation factor (SMF, which appears to be a fatty acid, 8,11,14-eicosatrienoic acid) have been described (Pacey and Bentley, 1992). In female polychaete worms, two substances may be involved in the initiation of oocyte maturation; a substance from the prostomium (prostomial maturation

hormone) which induces a coelomic maturation factor (CMF; Watson and Bentley, 1997). The role of the annelid endocrine system in vitellogenesis has yet to be confirmed (Porchet *et* al., 1989), although vitellin has been characterised in Nereis diversicolor (Bonnier, 1992). FMRFamide has been definitively identified in the polychaete *Nereis virens* (Krajniak and Price, 1990) and is believed to function as a neuropeptide. JH has been demonstrated to be an active inducer of settlement and metamorphosis in polychaete larvae (Biggers and Laufer, 1992).

Morphalactic hormones, which accelerate or inhibit growth and regeneration, are also known to exist. In addition hormones are involved in regulation of body weight, osmotic pressure, ion balance, blood sugar level. Ecdysteroids have been detected in annelids, including demonstration of the hydroxylation of ecdysone to 20-hydroxyecdysone (Barker *et al*., 1990).

Considerable attention has focused on the role of pheromones during reproductive processes in polychaete worms. Pheromones are believed to be involved in mate location, synchronization of the nuptial dance and gamete release and the role of pheromones in the reproductive processes of polychaetes has been discussed by Bentley and Pacey (1992) and Hardege *et al*. (1994). One such compound has been identified as 5-methyl-3-heptanone, which induces particular swimming patterns and induces males to release sperm (Zeeck *et* al., 1990). A second compound, 3,5-octadien-2-one, has been found to induce egg release by female worms (Zeeck *et al*., 1991). It has been demonstrated that volatile organic substances derived from crude oil will induce sexually mature male nereids to engage in spawning behaviour in the absence of females, culminating in the release of gametes into the water (Beckmann *et* al., 1995).

The consideration of chemical settlement cues falls outside the scope of this report. Many references to work on chemical cues involved in the induction of settlement and metamorphosis in marine larvae can be found in Jensen and Morse (1990).

2.5.6 Protochordata

The collective title protochordate is essentially a convenient term for a diverse assembly of animals sharing relatively few, although important, common features. Three subphyla comprise the protochordates - the Hemichordata, Urochordata and Cephalochordata. A summary of the endocrine tissues and products within these groups is provided by Tsuneki (1987) and a more detailed review of the functions of peptide neurohormones detected in the representative members of the Protochordata is undertaken by Thorndyke and Georges (1986). There are however no published reports on the occurrence of peptides in hemichordates.

The central nervous system and gastrointestinal tract have received most attention and immunocytochemical methods have been employed to demonstrate the existence of cross-reactivity with antisera raised to a wide range of vertebrate peptide hormones in these tissues. There is more limited information on the functional significance of many of these peptides.

In the cephalochordate Branchiostoma there is evidence for the presence of vertebrate-like steroids, progesterone, testosterone, estradiol-17β, and estrone and for effects of the vertebrate gonadotropin-releasing hormone (LHRH) on the synthesis of some of these steroids (Chang *et al*., 1985).

The distribution of neurohormonal peptides in protochordates has been studied extensively in recent years, yet relatively few species have been investigated - *Ascidiella aspersa, Ciona intestinalis, Styela plicata, S. clava* and *Branchiostoma lanceolatum.* These studies have been recently reviewed (Thorndyke, 1986; Thorndyke and Falkner 1985).

The relationship of the ascidian neural complex and to the vertebrate pituitary gland has been the subject of considerable debate since a possible homology was suggested over 100 years ago. With the description of a number of neurohormonal peptides in the complex, interest in the neural ganglion/gland as a pituitary homologue has re-emerged (Elwyn, 1937; Dodd and Dodd, 1966). There is little doubt that neurohormonal peptides are present in significant quantities in the Ascidian neural ganglion, but present evidence supports the idea that the neurosecretory cells of the ganglion are more comparable with neurosecretory neurones in invertebrates than with the pituitary complex of vertebrates.

Although information is available on the occurrence and distribution of neurohormonal peptides in protochordates little is known about their roles. Three of the better studied systems are described:

Reproductive cycles in *Ciona*

In tunicates a unique cycle of cellular activity in both neural ganglion and gland is closely correlated with lunar cycles and the ebb and flow of the tides. In a series of experiments the gland was excised from *Ciona* and it became apparent that the glandular cycle in *Ciona* reflects the production and release, during the mesenchymal phase, of a spawning inhibitory substance (SIS). The interaction of the ganglion, gland and ovary are complex and not fully understood (Georges, 1977; 1978).

Removal of the neural gland has no effect on events in the neural ganglion. It is the ovary which appears to play a part in the neuronal cycle (Georges, 1978); following removal of the ovary the ganglion cycle is disrupted but can be re-established on the introduction of ovarian tissue or extracts, or by the addition of steroids such as dehydroepiandrosterone and cortisol. Both external (tide and light) and internal stimuli are involved in the synchronisation of egg release. It is difficult to interpret the ganglion cycle in terms of changes in peptides. Of those neurohormonal peptides which have been described, only changes in the ACTH - like reactivity are correlated exactly to the cycling phenomena.

Reproduction in *Branchiostoma*

Preliminary results on *B. belcheri* show that high levels of progesterone and testosterone are present in both sexes while 17β-estradiol and estrone were only elevated in females (Chang *et al.* 1985). Subsequent experiments have explored the effect of luteinising hormone (LH), prolactin (PRL) and LHRH on both steroid levels and steroid production by the gonads of males and females. Administration of LHRH results in increased progesterone and estradiol levels in females, while human chorionic gonadotropin (hCG) enhanced progesterone production but estradiol did not. In males testosterone levels were increased, with LHRH showing higher potency than hCG. Ovine LH and PRL have also been shown to increase progesterone levels, with LH being more potent than PRL. Testosterone production is also stimulated by LH and PRL, with LH being more potent in males and prolactin in females.

These results are particularly interesting because for some time Hatscheks pit has been suggested as a likely homologue of the vertebrate pituitary gland (Tjoa and Welsch, 1974).

Gut secretions in Ascidians
Immunochemical studies suggest that the products of granulated endocrine cells in the alimentary canal of ascidians are immunochemically similar to polypeptide hormones such as secretin, bombesin and others which have established roles in vertebrate gastrointestinal physiology (Thornyke, 1986). Ultrastructural evidence also exists that the enzyme secreting cells may represent pre-pancreatic vertebrate zymogen cells (Thorndyke, 1977). It was established in *Styela clava* that certain vertebrate gastrointestinal secretions have significant stimulatory effect on stomach secretory activity. All forms of cholecystokinin (CCK) are able to stimulate enzyme secretion in vivo in a *Styela* bioassay, but *Styela* receptors are unable to discern between sulphated and non-sulphated CCK.

On the basis of current data it is difficult to assess whether there are a variety of receptors in *Styela* or a single receptor class. Notably all peptides which are active in *Styela* are thought to operate in vertebrates through a calcium-dependent second messenger.

There are a number of physiological processes for which it seem that peptides and/or amines may play a role :
Control of branchial cilia in ascidians
Contractile activity of body wall and siphons in ascidians and pelagic tunicates
Control of cardiac activity in ascidians

2.6 Conclusions

- It is clear from this brief survey of the endocrinology of the major invertebrate groups that utilisation of hormones to control and coordinate physiological and behavioural processes is common to all major invertebrate taxa.

- Neuropeptide signalling mechanisms, which utilise the peptide products of specialised neurosecretory cells, appear to be most predominant among the endocrine systems so far characterised in the invertebrates.

- However, ample evidence exists for the importance of non-peptide endocrine messengers in many groups (JH, ecdysteroids) although a functional role for vertebrate-type steroids has yet to be defined in most groups, other than perhaps the Echinodermata.

- All invertebrate groups must be considered Aat risk≅ or potentially susceptible to interference at a sub-lethal level by endocrine disrupting chemicals in the aquatic environment. To a certain extent this is already demonstrable in the application of certain

chemicals as pesticides, for example the JH analogues, which are known to operate via the endocrine system of the target organism.

- Given the complex and multi-functional nature of the role of many non-peptide hormones in invertebrates, the likely impact of interference at any particular locus within the endocrine system will be difficult to predict. It is unlikely that effects of disruption will be restricted to the reproductive system but rather will focus on any of the key invertebrate life stages and activities such as moulting, feeding, behaviour, reproduction and growth .

- Further difficulties in assessing effects lie in the diversity of the aquatic invertebrate biota, the varying degree to which the endocrine systems of different groups are characterised.

3. THE DETECTION AND ASSESSMENT OF ENDOCRINE DISRUPTING EFFECTS IN AQUATIC INVERTEBRATES

This Chapter will address the following points:

! are existing invertebrate test systems adequate to detect the effects of endocrine disrupting chemicals in invertebrates?

! which species of invertebrates are most appropriate for such tests?

! what end-points should be measured in such tests?

! is it possible to utilise the same organism for laboratory testing and environmental monitoring?

Before addressing these issues it is necessary to define the exact purpose for which test systems are required. Two main applications can be envisaged; the detection of chemicals with endocrine disrupting activity under controlled laboratory conditions (screening); the detection of endocrine disrupting effects in the natural environment (monitoring).

3.1 What type of laboratory test is most appropriate for the detection of chemicals with endocrine disrupting effects in invertebrates?

The most appropriate approaches to employ in testing chemicals for endocrine disrupting effects in aquatic invertebrates have recently been considered (Ankley *et al*., 1998; Tattersfield *et al*., 1997). Some significance was placed in these discussions on the ability of existing tests to identify compounds which disrupt the invertebrate endocrine system and it was concluded that in many cases a lack of understanding of the endocrinology of certain invertebrate groups hinders progress. Potential strategies for evaluating endocrine disruption in invertebrates will be examined, after consideration of the approaches currently being employed to evaluate endocrine disrupting chemicals in vertebrates.

3.1.1 Detecting endocrine-disrupting activity in vertebrates

A number of *in vitro* and *in vivo* test systems are currently available to facilitate the identification of compounds which interact with vertebrate steroid receptors and may therefore have potentially disruptive activity in vertebrate endocrine systems. Considerable interest has focused on the ability of chemicals to mimic estrogens (see for examples, McLachlan and Korach, 1995) and the testing of compounds for estrogenicity can be accomplished by a number of methods. These include

- direct assessment of the ability of the compound to bind to the estradiol receptor (Jobling *et al*., 1995);

- the use of fish hepatocyte suspensions in which the production of the yolk protein

vitellogenin is indicative of estrogenicity (Jobling and Sumpter, 1993);

- the measurement of the accumulation in cultured hepatocytes of mRNA encoding the estrogen receptor or vitellogenin (Flouriot *et al*., 1995);

- the utilisation of a yeast-based steroid hormone receptor gene transcription assay which employs yeast cells incorporating the human estrogen receptor together with response elements controlling a reporter gene (Routledge and Sumpter, 1996; Gaido *et al*., 1997);

- quantification of the effects of exposure to chemicals on uterine growth in rodents (Odum *et al*., 1997) or on a broader range of reproductive system parameters (O=Connor *et al*. 1997);

A similar methodology is beginning to be applied to the androgen receptor system (Kelce *et al*., 1995; Kelce and Wilson, 1997).

However, the use of receptor-binding assays, or tissue growth assays, while providing good evidence for the ability of a specific compound to interact with the endocrine system does not provide predictive information on the likely effects of such interaction at the whole animal level. In addition, *in vitro* assay systems suffer the disadvantage of not taking into account the scope for metabolism of xenobiotic compounds within the exposed animal, such that the endocrine system may be exposed, and be influenced by, a derivative of the original contaminant. Factors such as uptake, distribution and elimination are also unaccounted for.

Cellular and subcellular assay methods have been applied to vertebrate studies, not always or necessarily because of any particular insight provided by such methods, but largely because of the financial and practical difficulties inherent in carrying out full-scale *in vivo* investigations for every chemical of interest. Although whole animal methods have been utilised, for example the rat uterotrophic assay (Odum *et al*., 1997), these only provide information on effects of the test compound in specific tissues. This does not provide any indication, other than by inference, of the impact of the chemical on the function of the entire animal. For example, is the moderate elevation of blood vitellogenin levels seen in male rainbow trout exposed to river effluent indicative of longer-term effects on reproductive performance? Or is it functionally irrelevant (Sumpter, 1995)? While vitellogenin is an excellent biomarker for *exposure* to estrogenic compounds its measurement provides no information regarding the potential *impact* of those compounds. To resolve the question of impact will require a very significant commitment of time and effort.

These factors mean that the use of tissue, cellular, or sub-cellular assays to identify disruption of specific components of the endocrine system may not be the most appropriate approach to employ when considering the impact of potential disrupters on invertebrate endocrine systems.

Summary

! **The majority of test systems for endocrine disruptive chemicals in vertebrates are concerned with the detection of compounds with estrogenic activity**

! **Subcellular, cellular and organ-based assay systems provide evidence of hormone-like activity, and provide insights into mechanisms, but are not predictive of whole animal impacts**

3.1.2 Bioassays for invertebrate hormones: potential indicators of endocrine disruption?

Bioassay systems played an important role in early endocrinological studies of both vertebrates and invertebrates and numerous bioassays for hormonally active compounds have been developed in various invertebrate groups. In most cases this has been necessitated because many investigative methods now in widespread use (e.g. radioimmunoassay) were not available to researchers, or the chemical identity of the hormones of interest was not known, or purified hormone was unavailable, or the questions being addressed could be answered using a relatively unsophisticated approach. Invertebrates are particularly suited to this approach because of their lower level of organisation. They are more tolerant of extreme manipulations, such as the extirpation of entire body parts, or organs, or the ligaturing of sections of the body, than are vertebrates. Such approaches have been crucial to the advances in our understanding of the endocrinology of higher invertebrates.

Numerous bioassay techniques are referred to in the insect endocrinology literature. For example, bioassays for prothoracotropic hormone (PTTH) are available, most of which utilize lepidopteran pupae as test animals. All are similar in design, involving extirpation of the brain immediately after pupal ecdysis. Injection of PTTH activity into these pupae initiates metamorphosis to adult. Other positive responses that are more rapid can be employed (Williams,1968; Kobayashi. and Yamazaki, 1974). Assays for moulting hormones and juvenile hormones (JH) also exist (Thomson, 1974; Bjerke and Roller, 1974). A number of bioassays for JH are listed in Tobe and Feyereisen (1983). These include allatectomy followed by hormone replacement; transplantation of corpora allata (CA) to other stages or species followed by assessment of developmental and reproductive processes (e.g. the presence of structures associated with larval or adult stages at the next ecdysis), the nature and colour of the cuticle, the growth of oocytes in the adult female, supernumerary or intermediate ecdysis; CA volume or nucleocytoplasmic ratio or both; ultrastructural morphology of the CA, incorporation of radiolabelled isotopes as indicators of macromolecular synthesis. Bioassays for ecdysone exist and most are developments of puparium assays in which larvae are ligated 24h before expected pupation. The pupated anterior portion is cut off and the posterior part is used for the injection of test solutions. If these contain ecdysteroids at sufficient concentration the posterior part will pupate within 12-24h (Highnam and Hill, 1977). Many bioassay systems have been developed or exploited in the search for novel insecticidal compounds (Staal, 1986).

A similar situation applies to crustacea in that the extremely patterned and programmed development of crustaceans provides an ideal background against which alterations consequential to the introduction of hormonally active compounds may be readily identified. In common with other invertebrates, extreme physical manipulations of crustaceans are possible. The moult cycle, and larval development, provide straightforward means of assessing the ability of compounds to disrupt or alter this aspect of development in crustaceans (Koskela *et al*., 1992; Celestial and McKenney, 1994) and influences on the reproductive system may be detected by

determining relative rates of gonadal development or monitoring the presence of secondary sexual characteristics, such as oostegites (Fingerman, 1987). Bioassays continue to be developed to address specific requirements such as a means to assess the activity of ecdysteroids in a rapidly-responding membrane receptor system (Tomaschko *et al*., 1995). In this case the dilatation of the anterior oesophagus is measured in decapod crustaceans as proportional to the concentration of ecdysteroids present.

A number of bioassays for invertebrate hormones are described by Highnam and Hill (1977). However, much of the primary literature on bioassay systems for invertebrate endocrine studies pre-dates the establishment of the on-line databases used to prepare this report. These assays are reported in the literature as and when used but the primary literature is inaccessible to the search methods used in preparing this report. Because of constraints on time and manpower, we have been unable to prepare a source list by trawling of contemporary literature. However, as will be argued below, the use of bioassays/biomarkers to indicate interference at a specific locus within the endocrine system may not be the most effective way of detecting chemicals with disrupting activity in invertebrate systems.

Summary

- **A range of bioassays for compounds which display hormonal activity in invertebrates are available, but for a limited number of species**

- **Conventional bioassays can identify compounds possessing hormonal activity, but do not provide data on integrated effects of exposure**

3.1.3 Are specific bioassays an appropriate approach to the detection of endocrine disrupting chemicals in invertebrates?

As noted above (Sections 3.1.1 and 3.1.2), two approaches to identifying chemicals which disrupt the invertebrate endocrine system may be adopted. One option is the development and assembly of a suite of biomarkers to detect the presence of potential endocrine disrupting chemicals in invertebrates.

If it is assumed that endocrine disruption by chemicals of exogenous origin is feasible only in systems regulated by non-peptide effectors, which appears to be the position adopted by those working on vertebrate endocrine disruption, then chemicals likely to be of significance to invertebrates are those which act as agonists or antagonists of juvenile hormone (insects, nematodes?), ecdysteroids (insects, crustaceans, annelids?), methyl farnesoate (crustaceans), and vertebrate-type steroids (molluscs?, echinoderms, coelenterates?). The level of understanding regarding the function of these compounds at the receptor level has not yet reached that which permitted the development of the assay tools described above for vertebrate estrogen and androgen agonists and antagonists. Furthermore, the function of some of these compounds within the invertebrate endocrine system is yet to be fully defined, particularly the question of whether vertebrate-type steroids actually function hormonally in invertebrates. These factors would necessitate the utilisation of relatively crude *in vivo* bioassay systems, similar to those described for insects above and, given uncertainty about the role of certain compounds or identity of

certain hormones, specific bioassays for all the major hormone groups within each invertebrate taxon are unlikely to be available. If a suite of biomarkers or assay systems were developed, these would in all likelihood be incomplete and fragmentary in terms of coverage within the major invertebrate groupings. If comprehensive testing of chemicals already in use is envisaged this would be an untenable starting point.

However, it can be argued that seeking evidence of interference in specific well-defined components of the invertebrate endocrine system is an inappropriate approach. If the primary question being addressed is "*Can xenobiotic chemicals disrupt the normal function of invertebrate species by modulating the activity of the endocrine system?*" then the first step to identifying chemicals of potential interest should be demonstration that effects occur at a level of integration indicative of serious perturbation of normal performance. Having established that a chemical causes effects which are manifested at the whole animal level of organisation it is then appropriate to seek clarification of which locus/loci interference is occurring at, and the mode and mechanism of action of the interference. In adopting this approach, invertebrates offer several practical advantages compared with vertebrate species.

In contrast to vertebrates, the size, ease of maintenance, tolerance of handling and short life cycle of many invertebrates means that integrative studies, which evaluate the impact of potential toxicants on all aspects of the test organism, including behaviour, become feasible. This approach is, to a certain extent, already employed to assess the toxicology of compounds for regulatory purposes and has been adapted in a limited number of cases to examine the impact of potential endocrine disrupting chemicals on a limited range of species.

The potential problems associated with adopting a biomarker approach to the detection of endocrine disruption are exemplified in recent studies. It was demonstrated that acute, though not chronic, exposure to pentachlorophenol (PCP), which is a known endocrine disrupter in mammals, inhibited the conjugation of testosterone to glucose in *Daphnia magna* (Parks and LeBlanc, 1996). The authors suggested that toxicant-induced changes in steroid biotransformation might provide a biomarker of exposure to PCP and other endocrine disrupting chemicals. Subsequent work demonstrated that 4-nonylphenol, another known vertebrate endocrine-disruptor, also influenced the biometabolism of testosterone (Baldwin *et al*., 1997). However, although these data provide evidence for the ability of known hormone mimics to disrupt steroid metabolism in *Daphnia* there is no clear functional significance. As discussed in section 2.2.8 there is no firm evidence that vertebrate-type steroids play a role in the crustacean endocrine system and as yet there is no evidence that metabolism of the crustacean ecdysteroids would be affected by the processes observed. Therefore the functional significance of the effects observed in *Daphnia* cannot be definitively established, nor can a direct causal link to reproductive dysfunction be demonstrated.

Two concerns arise from adopting as a biomarker a response index which is sensitive to xenobiotics but has no demonstrable (or documented) role in growth, development, reproduction, or behaviour. First, it is possible that the biomarker will be activated in the absence of discernible effects at higher levels of organisation, for example, on reproduction. There is a danger that a screen based on this approach would be over-sensitive, detecting exposure to chemicals at levels below that at which a functional impact occurs. A second consideration is that the functional

effects of different chemicals which cause adverse changes in growth, moulting, reproduction or behaviour may be similar but the mechanistic basis underlying such effects may vary greatly between chemicals. Therefore, it seems unlikely that single, or even groups of, biomarkers might be developed which would provide confidence that exposure to endocrine disrupting chemicals could be detected in a given invertebrate species.

The alternative option is to employ a more robust bioassay test system which would allow the detection of compounds having substantial disruptive effects on whole animal function. As already discussed in earlier sections of this review, the role of the endocrine system of invertebrates is critical in the control and coordination of developmental processes, growth, moulting, reproduction and behaviour. It is reasonable to suggest that endocrine disrupting activity will be most readily detected in assay systems which utilise a response index which integrates these processes. Such a test could arguably be described as having a biomarker function (see 3.4), in that it indicates the presence of endocrine disrupting activity, but would extend the information obtained to include a definitive assessment of functional whole animal impact, on factors such as growth, reproductive performance, and behaviour.

Summary

- **There is a lack of basic knowledge regarding the function of the endocrine system in many invertebrate groups**

- **With some exceptions, the potential sites of action of endocrine disrupting chemicals in invertebrates have yet to be established**

- **The development of suitable biomarkers or screens, similar to those employed to detect estrogenic effects of xenobiotic compounds in vertebrates, for each major invertebrate group, and each potentially sensitive element of the endocrine system, is an unrealistic proposition**

- **To be of value in predicting effects of exposure and assessing likely impacts a functional relationship must be established between an indicator of exposure and subsequent effects - most existing biomarkers do not provide this link**

- **In contrast to vertebrate species, the size, ease of maintenance, tolerance of handling and short life cycle of many invertebrates means that integrative/holistic studies, which evaluate the impact of potential toxicants on all aspects of the test organism, including growth, reproduction and behaviour, are a practical proposition**

3.1.4 Should detection of effects encompass understanding of mechanisms?

The priority requirement is to establish whether invertebrate populations are at risk from endocrine-disrupting chemicals. Because of uncertainties regarding the potential of specific

chemicals to interact with the invertebrate endocrine system, and the exact locus of any such interference, an understanding of the mechanistic basis underlying any suspected endocrine disrupting effects in invertebrates must of necessity be divorced from the primary requirement to establish which chemicals exert adverse effects. As discussed above, this would exclude from a suite of suitable routine tests any *in vitro* or mechanistic assays in which the response criterion is at a level of organisation below the whole animal (e.g. receptor-binding studies, enzyme induction) and in which the whole-animal impact cannot be predicted with confidence.

If this approach is accepted then it must also be considered that lack of knowledge regarding the mode of action which underlies the adverse effects of a given chemical in integrated bioassays also reduces the possibility of being certain that the chemical in question is acting through disruption of endocrine processes as opposed to acting at sites other than the endocrine system.

The corollary of this is to pose the question whether it is necessary to discriminate between chemicals with regard to their mode of toxic action, providing that the test system employed is sensitive enough to detect sublethal changes which might arise as a consequence of interference in the endocrine system, in addition to more Aconventional≅ routes of toxicity.

There is therefore a strong case for ensuring that integrative, or holistic, test systems are sophisticated enough to detect effects which might arise through endocrine disruptive processes, in addition to more Aconventional≅ avenues of toxicity, rather than developing an additional battery of tests designed specifically to detect effects which might be attributed to endocrine disruption. One immediate benefit of this approach is that test systems likely to be useful in this context are already in widespread use and are comparatively Alow tech≅ relative to more sophisticated biochemical assay systems. In addition, the end-points of these tests are already accepted as determining limits on chemicals discharged to the environment whereas biomarker data are not acceptable in a regulatory framework, at least within the U.K..

A consideration of existing test methodologies suggests that these are in principle adequate to detect endocrine disrupting chemicals but that the range of species currently tested routinely may be inadequate. There is an added consideration that the sensitivity of different classes of invertebrates to compounds acting as endocrine disrupters may show significant variation, particularly if differences in the endocrine systems of invertebrates are reflected in the specificity of hormone AmimicsA. It is already established that very significant variation in species sensitivity to different classes of Aconventional≅ toxicants can occur (Vaal *et al*., 1997).

If the primary requirement of a testing programme is the detection of compounds which may be exerting sublethal effects via interference in invertebrate endocrine processes it is the view of the present authors that focusing effort on the mechanistic basis of endocrine disrupting effects in invertebrate groups is misplaced. Potential sites of interference are numerous and ill-defined and likely to remain so for many important invertebrate groups for the foreseeable future. While it is clearly of importance that the exact mechanisms by which compounds interfere with the endocrine systems of invertebrates is ultimately understood, and hence that research which addresses this is undertaken, such understanding is not a prerequisite for the detection of such effects.

Summary

- **The primary concerns associated with endocrine disrupting chemicals relate to adverse effects on the development, growth, reproductive and behavioural processes of exposed organisms**

- **The effects of exposure to endocrine disrupting chemicals can therefore be detected within the same test environments and utilising the same Aend-points≅ as effects arising from more Aconventional≅ routes of toxicity, with the attendant benefits of end-points which are acceptable in a regulatory context**

- **For the overall assessment of impact the mechanisms by which such effects are exerted are of secondary interest, however, knowledge of the endocrine mechanism may prove to be useful in assessing the sensitivity of the effect.**

- **There is a strong case for ensuring that existing integrative test systems are sensitive enough to detect effects which might arise through endocrine disruptive processes, in addition to more Aconventional≅ avenues of toxicity, rather than developing an additional battery of tests designed specifically to detect effects which might be attributed specifically to endocrine disruption.**

3.2 The application of existing toxicity testing protocols to the detection of endocrine disruption in invertebrates

The testing of new and existing compounds for lethal and sub-lethal effects in aquatic invertebrates is a well established element of the regulatory requirements and ever more sophisticated approaches to the assessment of the hazards associated with new compounds are under consideration (Cowan *et al*., 1995; Genoni, 1997).

Regulatory testing is carried out at the level of individual organisms, exposed individually or in small groups to the test compound for short or prolonged periods of time. The information obtained from such studies varies from data on survival only to a comprehensive assessment of effects of the test article on growth, development and reproduction, depending on the sophistication of the test conditions. Studies are generally carried out with representatives from a limited number of invertebrate species. A summary of representative organisms, test end-points, and source references is provided in Table 1 at the end of this section.

A more sophisticated test environment is provided by the use of *microcosm* systems, in which small groups of organisms may be maintained under semi-natural conditions designed to mimic normal habitat and natural diet (e.g. Kreutzweiser, 1997).

The use of *mesocosm* systems provides an approach to impact assessment which is intermediate between the laboratory based small-scale test system and direct environmental monitoring,

allowing control of experimental conditions but providing access to data on ecosystem, community, and population responses (e.g. Gillespie *et al*., 1996; Belanger *et al*., 1995).

Direct *environmental monitoring* involves the assessment of impact on assemblages of organisms at a site of natural exposure. It has been argued that this is the only approach which takes into account the complexities inherent in ecosystems (Zauke *et al*., 1996). The problems encountered with environmental monitoring of invertebrates are discussed by Schaeffer (1994).

Given the requirement for rapid results, inter-laboratory reproducibility, an acceptable throughput of compounds, and financial constraints, it is likely that small scale laboratory-based test systems will continue to be the most appropriate approach to testing compounds for effects. All three options will be discussed below in the context of detecting effects of chemicals which may act as endocrine disrupters in invertebrates.

3.2.1 The range of existing invertebrate toxicity testing systems

The utilisation of invertebrate species for testing purposes offers the opportunity to employ a top-down approach to assessing the toxicity of chemicals, in contrast to the situation in vertebrate testing, where of necessity a bottom-up approach must be adopted because of difficulties inherent in whole animal and multi-generation testing.

The toxicity of chemicals to aquatic invertebrates is currently assessed in two types of test systems. Short-term effects, usually on survival, are evaluated in an acute exposure system (#96 hours) and longer-term effects, on growth, reproductive performance and survival, are quantified in a chronic exposure system (∃10 days). Although the details may vary, in terms of species and exposure conditions, the intention is to identify a concentration of the substance which, under test conditions, has no observed effect (no observed effect concentration; NOEC) and which can be extrapolated to have no deleterious effect on organisms in the natural environment (predicted no effect concentration; PNEC). It has been speculated that the effect of toxic compounds on all species in a community can be extrapolated from data derived from a limited range of single species tests (Wagner and Løkke, 1991). A significant advantage offered by invertebrates, from the point of view of test protocols, is the accessibility in many species of the entire life cycle, over several generations in some cases, within a manageable time-scale.

To ensure comparability of results, a number of accepted Astandard≅ test protocols are in wide use, in particular those published by the Organisation for Economic Cooperation and Development (OECD, 1981), the American Society for Testing and Materials (ASTM**,** 1997) and the American Public Health Association (APHA, 1995). In addition, numerous publications and symposia have considered standardisation of aquatic invertebrate test systems (e.g. Buikema and Cairns, 1980; Murphy, 1980) and methods for the culture of many aquatic invertebrate species have been documented (e.g. Lawrence, 1981).

In addition to the testing which is carried out to satisfy regulatory requirements associated with the registration of new substances, there is an extensive body of literature describing studies in which the adverse effects of specific chemicals, or mixtures of chemicals, already in use have been evaluated in greater depth.

The acute and chronic test systems have been designed with the assumption that the chemicals under test may have an adverse impact on some aspect of the physiology or behaviour of the test organism which will be manifested as a change in survival, growth or reproductive performance. An understanding of the mechanism by which this occurs is not a prerequisite to obtaining useful data.

A summary of representative toxicological methodologies applicable to the major invertebrate groups is provided below, drawn from both the Astandard≅ suites of methods and from research papers. The literature concerning toxicity testing methods is very extensive and no attempt has been made to provide an exhaustive coverage. Review articles have been used to provide an access point to the literature and individual papers have been cited to illustrate specific methods or to provide a generic example of a specific method or approach.

3.2.2 Aquatic insects as test organisms

Toxicants disrupt the survival, growth, reproduction, and emergence behaviour of aquatic insects and it is end points associated with these processes which are normally assessed during test procedures. Clearly, these end points represent processes which it may be assumed are susceptible to interference by endocrine disrupting chemicals and would thus be suitable for detection of endocrine disruptive toxicants and/or the assessment of impacts.

The American Public Health Association (APHA) Standard Methods provides brief guidelines for the selection, preparation, collection and culture of aquatic insects for toxicity testing purposes (Section 8750; APHA, 1995e). The recommended species range for routine testing includes representative species from the stoneflies (Plecoptera), mayflies (Ephemeroptera), caddisflies (Trichoptera) and true flies (Diptera) and the procedures to be followed for assessment of short-term survival (96-168 h), survival for 5 - 60 days, adult emergence, and full life-cycle tests (90 - 120 days) are provided. The sophistication of the test environment employed is dependent on the duration of the test and which species is used and a wide range of response measures are accessible, including percentage of adults that emerge, sex distribution, incidence of incomplete emergence, adult length, weight and head capsule width, and number of mature eggs.

Other catalogues of test methods for aquatic insects are available. Murphy (1980) reviewed the use of immature stages of the Orders Ephemeroptera, Trichoptera, Plecoptera and Odonata (dragonflies) in toxicity testing and identified published guidelines for the holding, acclimating and culturing of representative species of these orders. These organisms have been used in acute and chronic toxicity test procedures with survival, growth and emergence success all being utilised as end points. Sensitivity to toxicants has been observed to be dependent on the instar used, the stage in the moult cycle and the season in which the animals were collected, all of which are clearly factors to be aware of when seeking evidence of endocrine disruption.

Some of the literature concerning test systems for the most commonly employed aquatic insect species is considered below.

Diptera: The Chironomidae

The ubiquity of chironomids (midges) within the aquatic environment, their tolerance to

manipulation, and the fact that adults will mate even while confined, has led to their adoption as a key test organism and many studies using these organisms have been reported. Eggs hatch within 3 days of deposition at 20^{o}C and there are four phases (instars) of larval growth of 4-7 days each followed by pupation and emergence as an adult. The sexes are readily distinguished. Standard methods are provided by the American Society for Testing and Materials for the use of *C. tentans* and *C. riparius* in the assessment of sediment-related toxicity (ASTM, 1997, E1706-95b). In these guidelines larval survival, larval growth and adult emergence are recommended end-points. In addition, egg masses can be collected and the effects of exposure on fecundity and hatch success can be assessed. Techniques for the maintenance of long-term laboratory populations of certain chironomid species are well-established and long-term exposure studies extending over one or more complete life cycles are therefore feasible. A detailed account of the biology, culture, and use in test procedures of *Chironomus tentans* is provided by Townsend *et al*. (1981). These authors discuss collection of individuals, isolation of eggs, and mass culture methods. Rearing techniques for a range of species including *C. tentans, C. thummi* (=*C. riparius*), *C. pallidivitatus* and *C. halophilus* are reviewed by Credland (1973) and Anderson (1980) reviews the life cycle of a Atypical≅ chironomid and describes laboratory methods for culture and for the execution of short-term, partial life-cycle and life-cycle toxicity tests. Methods for the continuous laboratory culture of *Chironomus riparius* are provided by McCahon and Pascoe (1988).

Numerous accounts of toxicity studies employing chironomids have been published. For example, details of a life cycle study in *Chironomus tentans* are provided by Liber *et al*. (1989). The range of data collected during this study encompassed growth, emergence success and fecundity. The performance of the offspring of ovipositing females was evaluated as was ovipositing efficiency of the females themselves with other effects being assessed independently for males and females. A similar approach has been used by others. In an examination of the toxicity of the alkylphenol 4-nonylphenol to *C. tentans* growth, fecundity, emergence, sex ratio and viability of the offspring were evaluated (Kahl *et al.*, 1997) with effects observed only on the survival of 20-day old larvae at the highest concentrations (200 $\mu g\ l^{-1}$). West *et al*. (1997) assessed egg deposition (size of egg masses) and hatching success in *C. tentans* using image analysis software. A detailed description is provided by Brown *et al*. (1996) and Stewart and Thompson (1995) of the conditions employed in studies designed to assess the impact of phthalate esters and fluoranthene respectively on emergence of the midge *Chironomus riparius*. In both studies, *Chironomus riparius* larvae were introduced into test vessels containing either control sediment or sediment together with toxicant. After development through four larval stages followed by pupation, the emerging adults were retained in traps and counted and sexed.

The occurrence of deformities in chironomid larvae has also been used as a response index in test systems. In a study by Dickman and Rygiel (1996) abnormal elements within the menta (mouthparts) were identified and quantified in larvae of various chironomid species and similar approaches have been employed by others (Hudson and Ciborowski, 1995).

The chironomid *C. tentans* has also been employed as an *in situ* test subject. Chambers containing the organisms were anchored to stream substrate in a manner allowing continuous water exchange and contact with the sediment layer (Chappie and Burton, 1997). Although in this case only survival was monitored for a period up to 4 weeks, more sophisticated response

indicators could have been selected and employed.

It has been demonstrated that different larval instars of *C. riparians* may be more, or less, resistant to the toxic effects of a given compound (Williams *et al*., 1986). The possibility that different developmental stages of invertebrate species display variable sensitivity to xenobiotics emphasises the need to employ full life-cycle testing.

Other Dipteran families
Because of the requirement for disease-related control measures for these organisms, toxicity tests utilising species of the family Culicidae and Simuliidae (mosquitos) have been developed and are listed by Murphy (1980).

Hemiptera and Coleoptera
Both adult and immature stages of these orders, the true bugs and beetles, have been employed in toxicological tests (Murphy, 1980) although end-points were restricted to mortality.

Ephemeroptera
Fremling and Mauck (1980) summarise methodology for the collection, culture and toxicity testing of mayfly nymphs together with a brief summary of the life history of the insects. Both acute and chronic tests are considered although the only end point discussed is mortality. Details on the biology, collection of different life stages, and generation of stock cultures for the mayfly *Hexagenia rigida* are provided by Friesen (1981a) and the collection and culture of this species is alos considered in great detail in the ASTM guidelines for the use of invertebrates in testing sediment-associated toxicity (ASTM, 1997, E1706-95b). *Hexagenia* has a life cycle comprising four stages; egg, nymph, subimago and imago (adult) and for short-term tests the end-point is mortality while over longer periods survival, growth, burrowing behaviour and moulting frequency can be monitored..

Trichoptera
Caddis flies have been used intermittently as test subjects. McCahon *et al*. (1989) describe a study in which a variety of response indices were examined in the cased caddis fly *Agapetus fuscipes* exposed to cadmium. Factors considered included case-building activity, aggregative behaviour and survival of both cased and uncased larvae at several instars. The net spinning activity displayed by some caddis flies has been utilised as a behavioural feature which is sensitive to disruption by pollutants, together with more conventional response indicators such as ventilation rate and locomotor activity (Gerhardt, 1996). The response indices assessed in a study of the effects of low dissolved oxygen levels on the caddis fly *Clistoronia magnifica* included egg hatch, larval development, moulting success, time of moulting, pupation and adult emergence (Nebeker *et al*., 1996).

Summary

- **A wide range of aquatic insects have been employed for toxicity testing with representatives of the Chironomidae being particularly widely used**

- **Existing test systems provide a broad range of response measures including short-**

and long-term survival, emergence success, sex ratio, growth, fecundity, hatching success, and occurrence of larval deformities with additional species-specific behavioural measures such as, burrowing, case building and net spinning

! **Aquatic insects are suitable organisms for *in situ* testing at impacted sites**

3.2.3 Crustaceans as test organisms

Toxic compounds have wide-ranging effects in crustaceans and response measures include egg hatchability, rate and success of moulting, swimming ability, survival, growth, and reproductive performance. Most of what little work has been done on the effects of endocrine disrupting chemicals on invertebrates has focused on the cladoceran *Daphnia sp.*

Entomostraca: copepods and cladocerans

A considerable proportion of Ainvertebrate toxicology≅ utilises a small range of Crustacea as test organisms. Among the most widely applied protocols is the OECD *Daphnia* sp., Acute Immobilisation Test and Reproduction Test (OECD Guideline for testing of chemicals 202: adopted 4 April 1984). This comprises two parts, the content of which are summarised below. *Daphnia magna* has been widely used in toxicity testing in part because of its large size and ready availability.

The acute immobilisation test (Part I: the OECD 24h EC50 acute immobilisation test) utilises healthy, laboratory bred *Daphnia magna* or any other suitable *Daphnia* species, of not more than 24h old, of known history (encompassing the breeding method and any pretreatment). Twenty animals are divided into four groups for each concentration of test article and control. Water temperature during the test is held at 18 - 22^{o}C and within 1^{o}C of the set point. The test can be carried out under a light-dark cycle although darkness is acceptable. Effects of the test article are quantified by determining the percentage of animals judged to be immobile at 24h and (optionally) 48h.

Similar test conditions are employed for the reproduction test [Part II: the OECD reproduction test (at least 14 days)]. At least five concentrations of the test article are employed, starting at or around the 24h EC50 determined in the immobilisation test and ending at approximately 1/100 of the 24h EC50 although, if necessary, lower concentrations can be tested. Toxicity of the test article is assessed by determining the total mortality, time of first production of young, the number of young born and signs of intoxication in toxicant-exposed individuals, compared with controls.

ASTM also publish guidelines for using *Daphnia magna* in life-cycle tests (ASTM, 1997, E1193-96). A similar test system is recommended by the ASTM in their guidelines for a life cycle test utilising *Ceriodaphnia dubia* (ASTM, 1997, E1295-89). As is the case for *Daphnia*, this organism is readily cultured in the laboratory. The study is designed to assess a variety of factors associated with the impact of test chemicals on *Ceriodaphnia* and employs neonates of less than 12 h old, placed individually into vessels containing dilution water or test solution. These test solutions may be renewed at 24h intervals depending on the chemical nature of the

test article. At renewal of the incubation medium, all offspring from each first generation *C. dubia* are counted. The test normally continues for 3 broods and at 25^{o}C this may only require 7 days. The date of death of each first generation *C. dubia* is recorded. The length and weight of each surviving organism may be recorded at the end of the study. Both first and second generation organisms are observed for abnormal development or behaviour, such as uncoordinated swimming.

Specific test procedures are outlined in the APHA Standards document (APHA, 1995d) for assessing survival, growth, and reproductive performance in *Daphnia* and these do not differ significantly from the OECD and ASTM methods.

Murphy (1980) provides an extensive list of references to culture techniques and toxicity test protocols for a variety of crustacea including *Daphnia magna, D. pulex, D. galeata mendotae, D. laevis, D. dubia, D. retrocurva, D. parvula, D. ambigua, D. catawba, Moina macropa, Scapholeberis mucronata, Simocephalus serrulatus, Ceriodaphnia reticulata* and *C. quadrangula.* The biology, collection and culture of *D. magna* and *D. pulex* are described in detail by Leonhard and Lawrence (1981). However, Buikema *et al*. (1980) raise questions regarding the supposed sensitivity of *Daphnia* to pollutants. The authors reviewed aspects of daphnid biology relevant to toxicity testing including nutrition, starvation, genetic stability, diseases, osmotic and ionic balance, species selection, culture health, test organism age, acclimation, test duration, temperature, light, loading factors, experimental end-points. The relative sensitivity of *Ceriodaphnia sp*. and *Daphnia sp*. to xenobiotics has been discussed by Versteeg et al. (1997) who also present details of the existing test methods appropriate to *Daphnia sp*. and *Ceriodaphnia sp*. Koivisto (1995) has questioned the ecological relevance of *Daphnia* as a test organism, suggesting that *D. magna* differs significantly from other freshwater zooplankton in size, habitat and life history. In particular, Koivisto suggests that as *D. magna* is more susceptible to predation because of its size, it is effectively excluded from habitats containing vertebrate predators. Therefore it is not a Arepresentative≅ cladoceran in terms of distribution, occurring mainly in ephemeral habitats such as small ponds and rock pools. Furthermore, large daphnids such as *Daphnia magna* (5-6 mm) produce smaller neonates than, for example *Ceriodaphnia sp.* (<1.5 mm) whose larger neonates mature more quickly. Versteeg *et al*. (1997) suggest that the acute and chronic toxicity of a broad range of chemicals are approximately similar for *Ceriodaphnia sp.* and *Daphnia sp.* and that toxicity test data from representatives of the two genera be considered equivalent.

Many research papers report procedures employed for chronic exposure tests using *Daphnia* and related species. The range of parameters commonly examined is demonstrated in a study of the effects of the organochlorine pesticide endosulfan on survival, growth and reproductive performance of *D. magna* in which mean total young per female, maximum number of broods, mean brood size, mean number of broods, and mean time to first reproduction were utilised as reproductive response indices (Fernandez-Casalderrey *et al*., 1993). A similar approach was employed in an evaluation of the effects of nonylphenol (a known endocrine disrupter in vertebrates) on *Daphnia*, in which the most sensitive response was found to be the mean number of live offspring per surviving parent, although the NOEC was one order of magnitude greater than reported environmental values (Comber *et al*., 1993). In a study examining effects of the estrogen diethylstilbestrol on *D. magna* effects on moulting frequency and fecundity were

observed at 0.5 mg l^{-1} (Baldwin *et al.*, 1995). In contrast, an assessment of the effects of phthalate esters on *D. magna* showed no adverse effects of exposure to phthalates (1 mg l^{-1}) on growth or reproduction, assessed as numbers of young produced and length of parents after 21 days (Brown *et al*., 1998). Effects of nonylphenol (at environmentally relevant concentrations of 10 - 100 µg l^{-1}) were evaluated on the production of three types of offspring by *D. galeata mendotae* (female, male and ephippial) when crowded, and variable effects on the production of each type of offspring and on the occurrence of deformed offspring were observed in nonylphenol-exposed individuals (Shurin and Dodson, 1997). These authors made the point that although it is the asexually reproducing stage of *Daphnia* which is normally utilised in test systems, the organism may be more susceptible to xenobiotics during sexual reproduction. Moulting was utilised as a response index in a study in which *D. magna* were exposed to a range of xenobiotics which are estrogenic to vertebrates (Zou and Fingerman, 1997). For some of these compounds, moulting was inhibited although at concentrations above those occurring in the environment. The mechanisms underlying the effects cited above are open to debate; as described in section 2.2.8, there is no defined functional role for vertebrate steroids in crustacea. Therefore the exact mode of action in crustacea of chemicals which function as steroid mimics in vertebrates, and indeed whether effects are exerted via the endocrine system, is unclear.

Filtration and ingestion (Fernandez-Casalderrey *et al*., 1994) and consumption and assimilation rates (Bodar *et al*., 1988) of *Daphnia* have been used as response indices in sub-lethal test systems as has the detailed analysis of swimming behaviour (Dodson and Hanazato, 1995) and early growth patterns of neonates (Hanazato, 1998).

A more unusual end point is the development of Ahelmets≅ by *Daphnia ambigua*, a response normally observed to occur as an antipredator device. Hanazato (1991) demonstrated that a range of pesticides could also induce the formation of these structures.

The utilisation of species of regional significance also receives consideration, for example in a study in which reproductive impairment in the Australian cladocerans *Ceriodaphnia dubia* and *Moinodaphnia macleayi* exposed to endosulfan was examined (Sunderam *et al*., 1994) and in attempts to find more appropriate representatives of regional fauna (Julli *et al*., 1990; Anderson-Carnahan *et al*., 1995). There are concerns that the toxicity data obtained under standard test conditions may not be wholly applicable to all situations; regional differences may occur as a consequence of variation in the sensitivity of native species compared to test species, and due to variation in local environmental conditions. Vaal *et al*. (1997) reported that species differences in sensitivity to toxicants were most marked for reactive and specifically acting compounds, a group which would include the mode of action of endocrine disrupting chemicals. A further complication, as Sunderam *et al*. (1994) make clear, over and above consideration of species response differences there can be considerable variation in published figures for the toxicity of chemicals tested on the same species at different laboratories. ASTM guidelines (ASTM, 1997, E1850-97) also note that Ain systems where surrogate species are not found, erroneous predictions might be obtained of environmental impact or water and sediment quality impairment based on toxicity tests using surrogate species.≅

Techniques for the culture and testing of representatives of the subclass Copepoda are also listed

by Murphy (1980). It is pointed out that these organisms do not reproduce by diploid parthenogenesis and cannibalism may make prolonged maintenance of stable cultures difficult. Dodson and Hanazato (1995) have speculated that the more flexible sex ratio apparent in Cladoceran species, as opposed to the 1:1 ratio in obligate sexually reproductive species (such as copepods) might make them more sensitive indicators of exposure to endocrine-disrupting chemicals. However, it might also be argued that copepods represent a model more similar to that of vertebrates and because deviation from the 1:1 ratio is rare, endocrine influences will be more obvious.

Summary

- **The cladoceran crustacean *Daphnia sp*., together with other related species, has been extensively utilised in regulatory and non-regulatory toxicity testing as a representative microcrustacean**

- **The organism is readily cultured and the range of responses to toxic challenge which are measurable includes growth (length, weight), intoxication, abnormal behaviour (e.g. swimming), mortality, time to first production of young, fecundity/ number of broods, type of offspring (female, male and ephippial), moulting frequency, helmet formation, filtration and ingestion rates. All these should be considered as potentially valuable indicators of exposure to endocrine-disrupting chemicals**

- **Despite the wide usage of *Daphnia sp*. for toxicological purposes, some concerns have been expressed regarding the degree to which *Daphnia* is an appropriate representative of freshwater zooplankton. In part because the role of steroid hormones in development and reproduction are not understood and available evidence suggests that compounds acting via the androgen and estrogen receptor do not affect the endocrine system of *Daphnia sp*. But also because it is not representative of a "typical" freshwater zooplankton.**

Malacostraca: mysids, amphipods, isopods, decapods

Although not so widely utilised as *Daphnia*, a number of other crustacean species have been employed in various toxicity testing systems.

Mysids, shrimp-like crustaceans, are widely employed as test subjects, exemplified in an ASTM protocol (ASTM, 1997, E1191-97: Standard guide for conducting life-cycle toxicity tests with saltwater mysids). This procedure provides information on the effect of the test article on selected species of mysids during continuous exposure from shortly after birth until the beginning of reproduction. Species in common use include *Mysidopsis bahia, M. bigelow* and *M. almyra.* Techniques for rearing mysids in the laboratory are well documented. Test organisms are collected within 24 h of release from the brood sac and are immediately exposed to the test article. When sexually mature (at 10-14 days old) the organisms are physically separated into pairs. The complete life cycle test ends when the last first-generation mysid dies. Live young are

counted and removed daily and the day of release is recorded. Measurement of the body length of surviving mysids is carried out. Both first and second generation mysids are observed for evidence of abnormal development and behaviour. Morphological examination of first generation mysids alive at the end of the test is desirable. In addition, data can be collected on the survival, development and behaviour of second generation mysids. In common with other chronic exposure tests which integrate effects on growth, development and reproduction, this system is likely to detect effects due to endocrine disruption.

The ASTM protocols have been thoroughly evaluated by Lussier *et al*. (1996) who recommend a particular combination of conditions (randomized sex ratios with larger test compartments and greater replicaton) as providing the most efficient and statistically powerful version of the test.

The American Public Health Association Standard Methods (APHA, 1995c,d) provides details of suggested test regimes for both short-term and partial or complete life-cycle tests employing either microcrustaceans (Section 8710; copepods and cladocerans; defined as planktonic, e.g. *Daphnia sp*.) or macrocrustaceans (Section 8720; mysids, amphipods, isopods, decapods). A wide range of freshwater and marine crustaceans are recommended as subjects for use in toxicity testing and procedures for their collection, maintenance, and culture are provided. Detailed information is provided for amphipods, crayfish, crabs, lobsters and shrimps. The ASTM also publish guidelines for the use of marine amphipods in assessing sediment toxicity (ASTM, 1997, E1367-92). It is likely that the same criteria currently employed to select appropriate species for toxicity testing will apply to the selection of species for the detection of endocrine-disrupting effects.

The use of marine crustacea in ecotoxicological test procedures was reviewed by Gentile *et al.* (1984). These authors indicated that larvae, juveniles and adults were the life stages most frequently used and that the trend was for the testing of larval stages to be favoured. The most commonly evaluated responses were mortality, developmental rates, physiological and behavioural effects. Detection of endocrine-disrupting effects is likely to require full life-cycle testing, rather than focusing on a single life stage - it is conceivable that the sensitivity of different life stages will vary. Recent studies on the marine copepod *Tisbe battagliai* (Harpacticoida) exemplify this approach. Lifecycle effects of the ecdysteroid 20-hydroxyecdysone and the estrogen diethylstilbestrol were assessed using survival, development, sex ratio and reproductive output as endpoints (Hutchinson *et al*., 1999a,b).

Murphy (1980) also lists methods for Malacostracan crustaceans including amphipods of the genus *Gammarus* which have been used extensively but are found to be generally more sensitive to non-specific test conditions than *Daphnia*. *Gammarus fossarum, G. tigrinus* and *G. pulex* have all been used. As is the case for the OECD, ASTM and APHA tests which are referred to above, chronic exposure/life history studies have been carried out in which evidence for deleterious effects over the entire life cycle are sought with the normal parameters of survival, reproductive success and growth being recorded. Methods for short-term, intermediate and long-term tests employing *Gammarus fasciatus, G. lacustris* and *G. pseudolimnaeus* are considered by Arthur (1980) and detailed accounts of the biology, collection and mass culture of the amphipods *Hyalella azteca* and *Gammarus lacustris* are provided by de March (1981a and 1981b). McCahon and Pascoe (1988) provide details on continuous laboratory culture methods for *G.*

Pulex and *Asellus aquaticus*.

Numerous research papers have been published which describe studies utilising crustaceans other than copepods and cladocerans and which employ response indices such as survival from hatch to megalops to crab (*Rhithropanopeus harrisii*; Celestial and McKenney, 1994); growth, date of brood release, brood size (*Mysidopsis bahia*; McKenney and Celestial, 1996); cover-seeking behaviour in juvenile crayfish (*Procambarus clarkii*; Misra *et al*., 1996); time spent on locomotion, periods of high activity, cleaning, ventilation (*Gammarus pulex;* Gerhardt, 1996). As is the case of for aquatic insects, detection of endocrine-disrupting activity is likely to require test procedures which integrate response measures for all critical processes including growth, development, reproduction and behaviour. It is clear from the studies cited above that existing test protocols which address all these issues are already established.

Crustacea are also suitable organisms for *in situ* test systems, in which groups of animals are maintained within enclosures anchored at a test site (e.g. Chappie and Burton, 1997).

Summary

- **Standard toxicity test protocols utilising macrocrustaceans are well-documented**

- **A wide range of response measures are employed in macrocrustacean tests including survival, growth, development, fecundity, locomotion, ventilatory activity, behaviour (e.g. cover-seeking, cleaning). All are likely to be important response measures in evaluating endocrine-disrupting effects**

- **Macrocrustacea are suitable organisms for *in situ* testing at impacted sites**

3.2.4 Molluscs as test organisms

The APHA Standard Methods (APHA, 1995b) and ASTM (ASTM, 1997, E724-94) provide details on recommended test procedures which utilise marine bivalve molluscs. The ASTM guidelines specify Pacific oysters (*Crassotrea gigas*), Eastern oysters (*C. virginica*), quahogs or hard clams (*Mercenaria mercenaria*), or blue mussels (*Mytilus edulis*) as suitable test subjects on the basis of availability, commercial significance and ease of handling. Full details on the maintenance of broodstock, spawning and fertilization are provided. The APHA text notes that standard methods for assessment of toxicity in freshwater molluscs are under development. The end points examined in mollusc-based tests include effects on fertilisation, embryonic development, growth, byssal thread secretion, and reproduction. Because adult bivalve molluscs close their shells in the presence of toxic or irritant compounds (Brown and Newell, 1972; Scott and Major, 1972; Delhaye and Cornet, 1975) they are not considered to be wholly suitable for test purposes. However, see below for exceptions to this.

Specific tests for which information is provided in the APHA manual include the oyster embryo test (in which normality of development is the response index, assessed as the proportion of fully shelled larvae), the oyster shell deposition test (in which relative shell growth is measured) and the oyster growth test for determination of chronic effects (in which weight is recorded at

intervals over several months exposure).

Benfield and Buikema (1980) list a number of toxicity studies undertaken with gastropod and bivalve molluscs, primarily simple LC50 tests, and the literature concerning the use of molluscs in ecotoxicological testing has been reviewed by Calabrese (1984). The author highlighted the relatively small range of species which are routinely employed in test systems and outlines methodology including the use of abnormal shell development or mortality in embryos (see above), larval survival, larval respiration, larval swimming behaviour and juvenile growth as response indicators. A more recent assessment of toxicity testing with marine molluscs is provided by Hunt and Anderson (1993) who provide a historical perspective and present information on the test species for which the widest validation of test methods is available. The species considered are the Pacific oyster, the Quahog clam, the blue mussel and the red abalone (*Haliotis rufescens*). Tests discussed include adult survival and growth, juvenile survival and behaviour, larval survival and growth, and embryo/larval survival and development. The marine gastropod red abalone has been utilised in a study of the effects of drilling muds on invertebrates (Raimondi *et al.*, 1997) in which the fertilisation success, larval development, larval settlement, larval survivorship and larval viability were assessed. The authors also provide full details of the collection and culture of this species of abalone. The swimming, grazing, growth and settling of mussel (*Mytilus edulis*) larvae were utilised as response indicators in a study on the effects of a surfactant (Hansen *et al*., 1997). Pelletier *et al*. (1997) provide a description of the use of two life stages (embryo/larval and juvenile) of the bivalve mollusc *Mulinia lateralis* in standard toxicity tests.

Details of the AD≅ embryo bioassay in which the proportion of fertilised oyster ova which develop into AD≅ embryos (a specific developmental stage at which the bivalve shell begins to form) are outlined by Abram (1993) together with a description of the collection of gametes from adult oysters.

Although most molluscan toxicity tests reported in the literature utilise marine species, Johnson *et al*. (1993) report a method for conducting tests using the early life stages of the freshwater mussel, *Anodonta imbecilis*. The authors provide methods for the culture of *A. imbecilis* and the testing of glochidia and recently transformed juveniles. Only acute test methods are described. Murphy (1980) reports that freshwater Gastropod molluscs have frequently been used in toxicology. Pulmonate gastropods, such as *Lymnaea stagnalis*, are readily cultured in the laboratory with development time from egg to adult in the region of 2-4 months and high fecundity. The standard criteria of survival, reproductive impairment and growth can be employed in test systems. Acute toxicity tests have been carried out on the egg, juvenile and adult stages of gastropod molluscs, usually over a 96h period. Chronic exposure studies have been described for a number of species including *Campeloma decisum, Physa integra, Helisoma trivolvis, L. stagnalis* and *L. palustris.* Details of the biology, collection, and mass culture of *H. trivolvis* are provided by Friesen (1981). Recent studies employing freshwater gastropods include that of Presing (1993) in which the toxicity of the insecticide K-Othrine to *L. stagnalis* was assessed using reproductive performance (number of eggs deposited) and survival of hatched juveniles as response indices. Baturo *et al*. (1995) describe a study in which the response of the gastropod *L. palustris* to atrazine and hexachlorobenzene was evaluated using growth and fecundity as response indicators. Activity levels have also been employed as a response measure

in *L. stagnalis*. In a study in which the effects of aluminium and lead on the activity of the snail were assessed time-lapse video recording was used to determine the total distance moved by the snails during given periods of time (Truscott *et al*., 1995).

Although adult bivalve molluscs are not generally considered suitable subjects for testing purposes, specific attributes of these stages have been employed as response indices in many published studies. For example, the filtration rate of adult zebra mussels (*Dreissena polymorpha*), determined as the decline in algal concentration in the bathing medium, has been employed an index of the chronic toxicity of copper and cadmium (Kraak *et al*., 1992). Abel (1976) provides details of a method employing the vital stain neutral red. Similar approaches to these have been employed by others with *Mytilus spp* (Manley, 1983; Grace and Gainey, 1987), and the green mussel *Perna viridis* (Krishnakumar *et al*., 1990). However, a note of caution was sounded by Reeders *et al*. (1989) who observed that under laboratory conditions abnormal patterns of filtration, associated with disturbance, were common in the mussel *D. polymorpha*.

For a full consideration of the issues associated with the imposex condition in molluscs, see section 4.1.

Summary

- **Standard methods are available for the maintenance of many molluscan species under laboratory conditions**

- **A wide range of toxicity test protocols are available for molluscs with many response indices identified. These encompass growth, development, reproduction and survival**

- **Specific indices include fertilisation success, embryonic development, adult growth (assessed by monitoring weight), byssal thread secretion, fertilisation success, normality of development (assessed as the proportion of fully shelled larvae), shell deposition (in which relative shell growth is measured), larval survival, larval development, larval respiration, larval swimming behaviour, larval settlement, juvenile survival, adult filtration rate**

3.2.5 Annelids, Nematodes, Echinoderms, Coelenterates

Annelids (segmented worms)

Both the OECD and ASTM guidelines include recommended protocols for assessing the toxicity of chemicals to annelids. The OECD Earthworm, Acute Toxicity Test (Guideline for testing of chemicals 207: adopted 4 April 1984) comprises a paper contact toxicity test, in which earthworms are exposed to the test compound on moist filter paper, and an artificial soil test, in which worms are maintained in artificial soil, to which the test compounds have been applied, for periods of 14 days. The end point for both tests is mortality.

The ASTM lists a Standard guide for conducting acute, chronic, and life-cycle aquatic toxicity tests with polychaetous annelids (ASTM, 1997, E1562-94). Four species are recommended: *Neanthes arenaceadentata, Capitella capitata, Ophryotrocha diadema, Dinophilus gyrociliatus,*

primarily to provide for comparability between studies, and extensive details on the culture of these organisms is provided. The acute test extends over a 96h period with mortality of the test organisms as the end point. The chronic test is conducted for a greater time period than 96h (total duration dependent on the life cycle of the organism used) with mortality again as the end point. The life-cycle test utilises juvenile polychaetes which are monitored until they mature and lay eggs. The number of embryos produced by each female is used as the response index. The duration of the study varies between 10 days and 3 months according to species. The tests can be extended to examine viability of the embryos. Published guidelines are also available for the evaluation of sediment toxicity to polychaete annelids (ASTM, 1997, E1611-94) in which growth and mortality are the primary response measures .

The American Public Health Association Standard Methods (1995a) also includes recommended procedures for the collection, culture and use in tests of marine polychaetes and marine and freshwater oligochaetes (Section 8150). The test procedures described utilise a sediment environment and the end points assessed can include growth rate, numbers of females forming eggs, numbers of females laying eggs and numbers of eggs and live offspring produced.

Reish (1980) describes procedures for culturing three species of polychaetous annelids from egg to egg under laboratory conditions. The methods described are for *Neanthes arenaceodentata, Capitella capitata* and *Ctenodrilus serratus.* Protocols for short-term and longer-term exposure experiments are described with mortality and reproductive performance being considered as response criteria. In a later review article Reish (1984) considers the species of polychaetes most widely used, the type of bioassays used, the degree to which assays have been standardised and the advantages and disadvantages of polychaetes as a test subject.

Efforts have been made by some authors to identify species more relevant to specific regions than those recommended by the standard test methods (e.g. Hickey and Martin, 1995). Hutchinson *et al*. (1995) present methods for the culture of *Platynereis dumerilii*, a polychaete indigenous to European waters and identify a number of end points for use in test procedures including fertilization rate, embryo-larval development, and larval survival.

Numerous recent studies report the use of oligochaete species in short- and long-term toxicity studies. For example West *et al*. (1997) describe a 28 day study with *Lumbriculus variegatus* in which survival and reproductive performance were assessed. *Tubifex tubifex* has also been employed for similar studies with similar end points (Roshon *et al*., 1995). Growth is also employed as a response index in studies utilising polychaetes (e.g. *Armandia brevis*; Rice *et al*., 1995).

Summary

- **Marine polychaetes, representative of the Annelida, can be cultured under laboratory conditions or collected from the natural environment**

- **Well-established test protocols exist for the assessment of toxic effects on polychaetes with end points including survival, growth, fertilization rate, fecundity, embryo-larval development, and larval survival**

Nematodes (round worms)

Although no standard test methods exist, the usefulness of the nematode *Caenorhabditis elegans* as an aquatic toxicity test subject has been evaluated. The use of nematodes in marine ecotoxicology testing was reviewed by Samoiloff and Bogaert (1984). The authors list a range of response indicators including cessation of feeding, mutagenesis, developmental arrest, and moulting and emphasise the fact that in comparison with other invertebrate groups, nematodes are relatively resistant to toxic effects. Attributes possessed by the organism include easy growth on solid or in liquid media, rapid growth, a short generation time and reproduction by either self- or cross-fertilisation (Williams and Dusenberry, 1990) and the tolerance of *C. elegans* to a wide range of environmental conditions make it a versatile test organism (Khanna *et al*., 1997). End points utilised under test conditions include survival, rate and direction of movement, feeding rate, fecundity and development (Donkin and Williams, 1995).

Summary

- **At least one species of nematode has been identified as a valuable and versatile test organisms**

- **A variety of response measures can be applied, including survival, feeding behaviour, locomotory behaviour and fecundity**

Echinoderms (star fish, sea urchins, sand dollars)

The earlier literature concerning the use of echinoderms in toxicity testing was reviewed by Kobayashi (1984). Information on tests involving gamete toxicity, embryo toxicity, developmental tests following exposure of egg and sperm to the test compound and larval development were provided. A more recent review of the use of echinoderms in toxicity testing is provided by Bay *et al*. (1993). The authors state that sea urchin and sand dollar gametes and embryos have been widely employed as test subjects and outline advantages and disadvantages associated with testing methods including assessment of sperm viability following exposure to toxicants, embryo exposure (in which abnormal or delayed development of the pluteus larva is assessed). The current ASTM guidelines (ASTM, 1997, E1563-95) describe the use of several echinoid species (Atlantic sea urchins, *Arbacia punctulata;* green sea urchins, *Strongylocentrotus droebachiensis;* Pacific purple sea urchins, *S. pururatus;* Pacific eccentric sand dollars, *Dendraster exentricus*) with end-points being mortality and quantification of abnormalities in embryo and larval development. Methods employing adult exposure are not widely used due to their large size and relative tolerance to toxicants. However, Bay *et al*. suggest that development of long-term test protocols utilising adults would allow an assessment of toxicant effects on growth and reproduction. Others have highlighted the advantages offered in the use of sea urchins in toxicity testing procedures as the high fecundity of individuals, embryogenesis which proceeds synchronously in seawater with high viability, fertilised eggs which develop into the larval stage within a short time period and the fact that live embryos may be observed microscopically without adverse effects on development (Nakajima *et al*., 1996). For examples of the use of developmental changes, sperm viability, and offspring survival/deformity as end

points see Trieff *et al*. (1995) and references therein. A comprehensive sea urchin test system is described by Dinnel *et al*. (1988) in which multiple end points are assessed. These include acute toxicity and behavioural modifications at the organism level, cytotoxicity and fertilization success at the gamete level, and developmental toxicity and genotoxicity at the embryo/larval stages, following exposure at the embryo stage, or *in vivo/in vitro* exposure of gametes. Pavilion (1988) provides a detailed account of various methods for employing sea urchin eggs and larvae in water quality assessment. Ramachandran *et al*. (1997) describe the use of the ASTM protocol in assessing toxicity in the sea urchin *Diadema setosum* in which gametes obtained from mildly electrically stimulated adults were combined and used to assess fertilization success (as presence of fertilization membrane) and embryo development at first cleavage, gastrulation and pluteus larvae stages.

The growth and survival of sand dollars, *Dendraster excentricus*, collected from the wild has been utilised in various assessments of sediment toxicity with growth response measures ranging from simple measurements of increments in diameter (Casillas *et al*., 1992; Rice *et al*., 1995) to additional determination of wet weight, and total DNA and protein content (Casillas *et al*., 1992).

Summary

- **The use of echinoid embryos in toxicity testing is well established**

- **Response measures include growth, effects on gamete viability, embryo survival, developmental assessment following exposure of egg and sperm to the test compound and larval development (identification of abnormalities or delays) during exposure**

Coelenterates (jelly fish, sea anemones, corals, hydroids)

The toxicological tests which utilise marine coelenterates have been reviewed by Stebbing and Brown (1984). Included among the range of organisms considered were representatives of the Scyphozoa, Hydrozoa, and Anthozoa. The authors examined the features of each group which could be considered advantageous or disadvantageous with regard to the requirements of test procedures. The sessile hydroid stages were considered easier test subjects than the medusae of hydroids with a similar situation pertaining to the Scyphozoa in which the sessile polyp stage is most suitable. More recent literature includes many studies which focus on animals from these groups. Methods for the culture of Hydrozoa have been described and the organisms are considered to have great potential as a standard test subject. Peters *et al.* (1991) describe the use of *Hydra littoralis* in acute toxicity tests. Corals have also been employed in published toxicity test procedures. The brown cup coral (*Paracyathus stearnsii*) was used in a study on the effects of drilling muds on marine invertebrates (Raimondi *et al*., 1997) in which the survival and extent of tissue loss of individuals was employed as the response index.

Summary

- **The use of coelenterates in toxicity testing is less widespread than the use of other invertebrate groups but is feasible and published methods exist**

3.2.6 Other invertebrate groups

Murphy (1980) reports instances of the use of freshwater leeches (Hirudinea) in toxicity tests although the mobility of these organisms represents a problem under laboratory conditions. Culture techniques have been reported.

Culture techniques are available for triclads (Murphy, 1980). Some acute toxicity tests have been reported but because of the long maturation period between egg and adult full life cycle tests may be impractical.

An ASTM protocol has been published for the use of the freshwater rotifer *Brachionus calicyflorus* with modifications provided for the use of the marine/brackish water species *B. plicatilis* (ASTM, 1997, E1440-91). However, mortality is the only end-point described for these tests. The wider use of rotifers in ecotoxicology is extensively reviewed by Snell and Janssen (1995). These authors note that the use of rotifers in ecotoxicological studies has increased, due largely to a recognition of the critical role of rotifers in freshwater communities; the ease with which measurements of mortality and reproduction can be quantified; the usefulness of the resting cyst stage; and the existence of reliable, standardized protocols. The authors describe studies in which mortality, reproduction (including rate of increase, maximum population size, frequency and amplitude of population oscillations, life expectancy, generation time) and behaviour (swimming activity, feeding) have been employed as end-points.

Table 1. A summary of representative test organisms from each major invertebrate group together with the end-points utilised in toxicity testing and source references.

Phylum	**Typical species**	**Documented end-points**	**Representative references (see text for additional references)**
Insecta	*Chironomus tentans* *C. riparius* (Chironomidae)	larval sex ratio, larval survival, larval growth, adult emergence, fecundity, occurrence of deformities	ASTM E1706-95b (1997) Brown *et al*. (1996) Dickman and Rygiel (1996) Kahl *et al*. (1997) Stewart and Thompson (1995) West *et al*. (1997)
	Hexagenia rigida (Ephemeroptera)	survival, growth, burrowing behaviour and moulting frequency	ASTM E1706-95b (1997)
	Agapetus fuscipes *Clistoronia magnifica* (Trichoptera)	case-building activity, aggregative behaviour and survival of both cased and uncased larvae at several instars, net-spinning activity, ventilation rate, locomotor activity, egg hatch, larval development, moulting success, time of moulting, pupation and adult emergence	Gerhardt (1996) McCahon *et al*. (1989) Nebeker *et al*. (1996)
Crustacea	*Daphnia magna* *Ceriodaphnia dubia* (Entomostraca)	total mortality, growth, time of first production of young, mean brood size, mean number of broods, signs of intoxication, abnormal development or behaviour, moulting frequency, filtration and ingestion, consumption and assimilation rates	OECD Guideline for testing of chemicals 202 ASTM E1193-96 (1997) ASTM E1295-89 (1997) APHA (1995d) Baldwin *et al.* (1995) Bodar *et al*. (1988) Fernandez-Casalderrey *et al*. (1993; 1994) Versteeg *et al*. (1997)
	Mysidopsis bahia *Gammarus spp.* *Rhithropanopeus harrisii* (Malacostraca)	survival, growth, development, fecundity, locomotion, ventilatory activity, behaviour (e.g. cover-seeking, cleaning)	ASTM E1191-97 (1997) ASTM E1367-92 (1997) APHA (1995c,d) Celestial and McKenney, (1994) Gentile *et al.* (1984) Gerhardt (1996) McKenney and Celestial (1996)
Mollusca	*Crassotrea spp.* *Mytilus edulis*	fertilisation success, embryonic development,	APHA (1995b) ASTM E724-94 91997)

	Lymnaea stagnalis	growth, larval survival, larval respiration, larval swimming behaviour, larval settlement, adult survival and growth, byssal thread secretion, filtration rate	Abram (1993) Baturo *et al*. (1995) Hansen *et al*., (1997) Hunt and Anderson (1993) Kraak *et al*. (1992) Presing (1993) Raimondi *et al.* (1997)
Annelida	*Neanthes arenaceadentata*, *Capitella capitata*	mortality, growth, numbers of egg-laying females, fecundity, embryo viability,	ASTM E1562-94 (1997) ASTM E1611-94 (1997) APHA (1995a) Reish (1984) West *et al*. (1997)
Nematoda	*Caenorhabditis elegans*	survival, feeding rate,fecundity, rate and direction of movement, mutagenesis, developmental arrest, moulting	Donkin and Williams (1995) Khanna *et al*. (1997) Samoiloff and Bogaert (1984) Williams and Dusenberry (1990)
Echinodermata	*Arbacia punctulata* *Strongylocentrotus droebachiensis* *Dendraster exentricus*	growth, gamete viability, embryo survival, developmental abnormalities	ASTM E1563-95 (1997) Casillas *et al*. (1992) Dinnel *et al*. (1988) Nakajima *et al*. (1996) Ramachandran *et al*. (1997) Trieff *et al*. (1995)
Coelenterata	*Hydra littoralis* various others	mortality, tissue loss	Peters *et al.* (1991) Raimondi *et al*. (1997) Stebbing and Brown (1984)
Rotifera	*Brachionus calicyflorus* *B. plicatilis*	mortality, reproduction (rate of population increase, maximum population size, frequency and amplitude of population oscillations, life expectancy, generation time) and behaviour (swimming activity, feeding)	ASTM E1440-91 (1997) Snell and Janssen (1995)

3.3 Mesocosms and microcosms

3.3.1 Mesocosms

The point has been made by many authors that standard lab-based toxicity tests, even chronic tests, do not accurately reflect the exposure times or conditions that may apply under natural conditions or that the response of single species in laboratory tests will not equate to the response of an assemblage of different species (Crossland and La Pointe, 1992; Kreutzweiser *et al*., 1994). Crane (1997) provides a brief summary of the rationale for employing a mesocosm scale approach to the evaluation of toxic effects in aquatic environments. Mesocosm systems, which are experimental enclosures containing a stable assemblage of representative elements of the natural ecosystem being modelled, offer the advantage of providing information on effects of contaminants at the population, community and ecosystem levels of organisation under conditions closely mimicking those in the natural environment (Boyle and Fairchild, 1997). The mesocosm approach also provides information on Aindirect≅ effects on biota other than target species (Belanger *et al*., 1995); subtle effects (e.g. on behaviour) on one or more component of the species assemblage, which may not be detected by direct assessment may nonetheless influence other elements of the assemblage in turn. No Astandard methods≅ exist for the design and application of mesocosms to invertebrate toxicity but numerous studies are reported in the literature. Those cited here are presented as examples and no attempt has been made to provide a comprehensive overview of this subject. For thorough reviews of the field the reader is referred to Graney *et al*. (1993), Grice and Reeve (1982), and Hill *et al*. (1994).

Mesocosms may be constructed to represent a range of different habitats including streams (e.g. Dorn *et al*., 1997; Gillespie *et al*., 1996, 1997) in which factors such as flow rate, rate and concentration of contaminant addition etc. can be closely controlled. Assemblages of appropriate species can be obtained by placing trays containing artificial substrate in suitable sites. The macroinvertebrates which colonise the trays reflect the fauna of the natural substrate and once colonised, the trays can be transferred to a laboratory-based stream system (Belanger *et al*., 1995; Kiffney and Clements, 1996). Alternatively, sediments can be physically transferred from a natural site to the enclosure (e.g. Giddings *et al*., 1996). After acclimation followed by exposure to the substances under test, the invertebrates can be retrieved from the artificial channels and quantified. Subtle behavioural responses such as Adrift≅, which could not be evaluated under laboratory conditions, can be assessed in mesocosms (Kreutzweiser *et al*., 1994). As noted above, indirect effects can be detected. In a prolonged (55 week) study of the impact of an insecticide on aquatic invertebrates, reductions in insect and crustacean abundance were accompanied by increases in molluscs and oligochaetes (van den Brink *et al*., 1996). In surfactant-treated stream mesocosms an increased heterotrophic periphyton biomass supported increased densities of gastropods and oligochaetes relative to untreated streams (Belanger *et al*., 1995).

A simpler alternative to the construction of purpose-built mesocosm systems is the use of enclosures within natural water bodies. For an example of the application of such a system see Peither *et al.* (1996) who used enclosures to assess direct and indirect effects of lindane on a zooplankton community . The advantages and disadvantages of enclosures are discussed by Liber (1994) who suggests that the main advantages of enclosures is their relatively low cost and

high ecological Arealism≅ while observing that problems can arise associated with sustaining adequate circulation, leakage, and possible heterogeneity of the enclosed communities.

The mesocosm approach is not restricted to freshwater environments. A complex estuarine mesocosm has recently been described (Lauth *et al*., 1996). Mesocosms have also been used to maintain populations of marine invertebrate larvae, specifically veligers of the gastropod conch *Strombus gigas* (Davis *et al*., 1996), larvae of the Atlantic mud crab *Panopeus herbstii* (Epifanio *et al*., 1991), echinoderm larvae (the starfish *Acanthaster planci*; Olson, 1987), the copepod *Acartia hudsonica* (Bollens and Stearns, 1992), and to study factors affecting the distribution and settlement of giant scallop larvae (*Placopecten magellanicus*; Gallager *et al*., 1996; Pearce *et al*., 1996) with obvious potential toxicity testing applications. Marine mesocosms have been employed in a study on the effects of a detergent on mussel larvae (*Mytilus edulis*; Hansen *et al*., 1997).

Major drawbacks associated with the use of mesocosm systems in impact assessment are the complexity of the data produced and the lack of reproducibility between systems (Crane, 1997). The most appropriate approaches to the evaluation of mesocosm study results are discussed by Shaw and Manning (1996). Crane (1997) also highlights the difficulties in using mesocosm derived results as the basis for predicting effects in the natural environment. Mesocosm test systems are unlikely to offer an acceptable routine method for the assessment of the effects of potential endocrine disrupting chemicals in invertebrates but do provide a means to carry out sophisticated impact evaluation where appropriate.

Summary

- **Mesocosm systems offer the best simulation of effects of pollutants in a natural environment on an assemblage of species**
- **The detection of subtle, indirect, effects of pollutant exposure is possible**
- **The major drawback to the employment of mesocosms lies in their complexity, both in terms of establishing and maintaining reproducible environments and in the interpretation of data**

3.3.2 Microcosms

Microcosms do not share the problem of replicability encountered with mesocosms to the same extent because such systems are simpler, with fewer component elements, are generally operated at a smaller scale, and under more controllable environmental conditions (e.g. Swartzman *et al*., 1990). In microcosms, community production, respiration and other biotic factors may be measured for the microcosm as a whole, providing an integrated picture of the impact of the toxicant on the community (e.g. Scanferlato and Cairns, 1990) or the effects on specific elements of the microcosm, such as species abundance, may be evaluated (Breneman and Pontasch, 1994). The range of end points which it is possible to determine using microcosm systems are discussed by Niederlehner and Cairns (1994) and include indicators at the level of energetics, nutrient

cycling and community structure. The ASTM publish a standardised protocol for microcosm studies (ASTM, 1997, E1366-96). The value of this system in obtaining data on the multispecies impact of a toxicant is discussed by Taub (1997).

Summary

- **Microcosms offer a compromise between the oversimplification of standard single species *in vitro* testing and the complexities inherent in mesocosm studies**

- **The microcosm remains too complex to employ as a routine test procedure when testing large numbers of potential toxicants**

3.4 Biomarkers of exposure to endocrine disrupters in invertebrates

Biomarkers may be defined as biochemical, cellular, physiological or behavioral responses of organisms that can be detected at the level of whole organisms or in tissue samples, urine or faeces that signal that exposure to anthropogenic chemicals or radiation has occurred. A range of biomarkers exist; some signal exposure alone while others are non-specific markers of adverse effect (Depledge, 1993). It is generally agreed that suites of biomarkers should be employed in field studies to enhance our ability to interpret the impact of pollutant exposure on the organism in question. The ideal biomarker is one which signals exposure to a specific type of chemical, exhibits a clear exposure-response relationship, and in which the magnitude of response is related to the degree of impairment of some parameter of reproductive health. Interestingly, the biomarker that most closely fulfills these requirements is the condition of imposex in molluscs (see 4.1). However, the example of the imposex condition is an exception at this time - similarly unequivocal biomarkers of endocrine disruption are not yet available for the majority of invertebrate groups. It must also be borne in mind that the imposex condition is a specific response to interference at a specific locus within the reproductive system of a single invertebrate group and does not provide a generally applicable biomarker; molluscan reproductive activity could be disrupted in a number of ways, the processess leading to the imposex condition represent just one of these.

Therefore, currently the application of the biomarker approach to the detection of endocrine disruption in invertebrates is constrained by a lack of knowledge regarding the identity of potentially disruptive chemicals and uncertainty regarding sites of action.

3.5 Assessment of the impact of effluents on natural populations of invertebrates

The biological assessment of anthropogenic impact on water bodies is a field of research which is too extensive to cover comprehensively within the confines of this report. Only the generalities and principles underlying the assessment of contaminant impact will be considered.

In principle, assessment of water quality may be achieved by two methods. In the first, an *in situ* comparison of the biota at impacted and unimpacted sites may be carried out. The problems inherent in obtaining suitable data, from the point of view of identifying Apristine≅ sites, and taking into account factors which affect the aquatic environment by routes other than changes in water quality (e.g. habitat alteration), are discussed by Chessman (1995). An evaluation of different analytical approaches to assessing invertebrate responses to a variety of human activities impacting on streams is provided by Fore *et al*. (1996).

The second approach involves an assessment of the effluent or receiving water quality by a controllable acute or chronic toxicity test procedure, either carried out in enclosures at the test sites or carried out under laboratory conditions using water collected from the test sites (e.g. Stewart, 1996). The toxic effects of municipal sewage effluents were assessed using this technique by Schroder *et al*. (1991) who examined the acute and chronic toxicity, and effects on reproduction, of a range of effluents on *D. magna* and *C. dubia*.

Reports of studies which have employed assessment of the macroinvertebrate community as a measure of effluent impact are provided by Wright *et al*. (1995) and similar studies on invertebrate species assemblages have been carried out in impacted/unimpacted marine or brackish environments (e.g. Simenstad *et al*., 1996). In these studies a wide range of benthic and epibenthic species are identified to class or order and quantified. Using a similar approach, the impact of increasing sewage effluent discharge in the Tyne estuary was assessed in part by consideration of the abundance of certain benthic invertebrate species (Hall *et al*., 1997). A comprehensive account of various approaches to biomonitoring using macroinvertebrates is provided by papers presented in Rosenberg and Resh (1993).

Summary

- **Numerous accounts are available of the assessment of effluent impact; either *in situ* by the characterisation and quantification of the local invertebrate species assemblage, or by introducing enclosures containing test organisms; or by the laboratory exposure of test organisms to water samples collected from the study site**

- **It is the study of *in situ* effects on invertebrate populations which will be most important in establishing the actual impact of endocrine disrupting chemicals on invertebrate populations**

3.6 Conclusions

In conclusion, the questions posed at the beginning of this Chapter can be answered as follows.

! **are existing invertebrate test systems adequate to detect the effects of endocrine disrupting chemicals in invertebrates?**

- Yes, a wide range of invertebrate toxicity test systems, covering all the major invertebrate groups has been well documented and are in widespread use. Freshwater test protocols are available for representatives of the Insecta, Mollusca, Crustacea and Annelida. Marine test systems have been described for Mollusca, Crustacea, Echinodermata, Coelenterata and Annelida.

- Longer-term, multi-life-cycle tests offer scope for evaluating the effects of potentially endocrine disruptive chemicals on survival, growth, developmental processes, reproduction and behaviour and therefore encompass the range of processes likely to be susceptible to interference by endocrine-disrupting chemicals.

- 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 end-points of, or carrying out more detailed assessments in, existing test protocols.

! **which species of invertebrates are most appropriate for such tests?**

- A range of invertebrate species from all the major taxonomic groups have been employed in toxicity tests. The species most appropriate for tests designed to detect endocrine disruption are those which it is realistic to use under laboratory conditions (i.e. can be collected and reared or maintained satisfactorily) and which reflect, or are comparable to, the biota which are indigenous to the regions in which the test compounds may enter the environment. These criteria are already employed to select the invertebrate species used in toxicity testing.

- It should also be noted that the study of endocrine disruption within invertebrate animals is a field which is in very early stages of development. It is not possible within the context of this report, and without wider consideration and discussion, to prescribe specific assay systems, or the use of particular test organisms.

- The endocrine systems of different invertebrate phyla utilise different chemicals as hormones

(see Chapter 2) and each phylum may therefore be affected by different chemical species. Thus, it is unlikely that a single representative invertebrate test which will be identified which can be used as being indicative of endocrine-related effects on invertebrates in general.

- Several authors have alluded to the importance of between-species differences in sensitivity to toxic chemicals. It is not known whether such inter-species variation in effects will apply to chemicals acting via disruption of the endocrine system but the selection of a Arepresentative≅ test species for each major group may be a practical necessity.

! **what end-points should be measured in such tests?**

- The end points which should be measured are those which allow the detection of interference in processes under endocrine control or influence. Therefore critical parameters are growth, development (the occurrence of abnormalities, interference in moult patterns, increased sensitivity related to different developmental stages), reproductive performance (gamete quality, fecundity, behaviour), and behaviour (locomotion, feeding, reproductive behaviour).

- These response indicators are already widely used to assess toxicity. Given the limited understanding of endocrine disruption in invertebrates it is not yet possible to be prescriptive regarding the exact parameters which should be assessed in order to ensure that effects are detected.

- It must also be borne in mind that effects on all these processes can occur via mechanisms which cannot be classified as endocrine disruption. Therefore, confirmation of mechanisms of toxic effect is desirable.

! **is it possible to utilise the same organism for laboratory testing and environmental monitoring?**

- In many cases, yes. See Chapter 6 for a discussion of the choice of sentinel species. Monitoring of invertebrate populations and communities in the aquatic environment is a well-established technique for the evaluation of xenobiotic impacts. This approach is therefore an appropriate means to assess the impact of endocrine disrupting chemicals at higher levels of trophic integration.

- However, it might be argued that in the natural environment, where organisms are exposed to a Acocktail≅ of xenobiotic compounds, distinguishing between the effects of chemicals acting via the endocrine system of invertebrates, and those acting via other pathways will be difficult.

3.7 Research priorities

The potential of certain chemicals, widely distributed within the environment, to act as hormone mimics in vertebrates has led to concerns that subtle but nonetheless adverse effects of these chemicals may be manifested in exposed individuals. Most of the effects identified to date are linked to abnormalities within the reproductive system. It is assumed that chemicals exerting endocrine disruptive effects have escaped detection during regulatory testing because the effects are subtle and the affected processes are not always examined as part of the response measures. One factor which has facilitated study and understanding of the phenomenon of endocrine disruption in vertebrates is the fact that all vertebrates, from fishes through to primates, share a common endocrine system, albeit with some species specific variation; for example, gonadal steroids in all vertebrates share closely related (in some cases identical) structures and roles. In contrast, an understanding of endocrine disruption in invertebrates requires that the considerable diversity of endocrine systems between the major invertebrate phyla is somehow accomodated.

It is known that adverse effects may occur via disruption of endocrine processes in invertebrate species either intentionally (e.g via pesticides) or unintentionally (e.g. TBT exposure). What is not yet known is the extent to which invertebrate populations are at risk to the adverse effects of chemicals acting via the endocrine system. The major issue which must be discussed before research priorities can be defined is whether it is necessary to distinguish endocrine-disrupting chemicals from chemicals which exert adverse effects via other mechanisms.

Many of the existing invertebrate toxicity testing protocols provide data on all the major processes which might be impacted by endocrine-disrupting chemicals, to a potentially greater extent than is the case for vertebrates. It is likely that thorough testing with existing invertebrate protocols would detect chemicals which exert adverse effects via the endocrine system. However, existing protocols tend not to provide data on the mechanistic basis of observed toxic effects.

What must be determined is whether it is appropriate to instigate a test regime (or continue with existing test strategies) which identifies effects without necessarily identifying the route by which those effects occur, or whether it is important to discriminate between compounds which act via the endocrine system and those which do not. 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. The most appropriate use of 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.

4. EVIDENCE FOR ENDOCRINE DISRUPTION IN INVERTEBRATES

A large and diverse range of chemicals is suspected of causing disruption of endocrine function in vertebrates (e.g. Colborn *et al*, 1993; Foster, 1995; Jobling *et al*. 1995; Montagnani *et al*.1996). Cooper and Kavlock (1997) observe that relatively few cases of contaminant effects on vertebrate reproductive processes can be confidently ascribed to endocrine disruption. They also suggest that the term Aendocrine disruption≅ has been applied to cases in which the biological basis for such effects has not been established. Caution is therefore needed in ascribing adverse effects of contaminant exposure to disruption of endocrine processes in vertebrates. This is of even greater relevance when assessing possible effects of contaminants on the endocrine systems 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. Some of the chemicals implicated in the disruption of vertebrate endocrine function also appear to have a similarly disrupting influence on invertebrate endocrine activity and chemicals not identified as having an effect in vertebrates may well impact on hormonal activities that are unique to particular groups of invertebrates.

Jobling *et al*. (1995) estimated that there are probably in the order of 60,000 organic micropollutants that occur in fresh-water from sources ranging from domestic and industrial effluents and leachates from solid waste disposal sites, to agricultural and urban run-off and atmospheric fall-out. Of this total, probably only about 3,000 have been identified. Since about 1945 large amounts of some estrogenic chemicals have been released into the environment, including well-known examples such as the insecticides, DDT and its metabolites and many of the polychlorinated biphenyls (PCBs). Chemicals originating from the plastics and detergent industries, such as alkylphenols, have also been shown to be estrogenic and there is evidence from various studies that these chemicals may have deleterious effects on wildlife populations. (Jobling *et al*. 1995).

Sewage, one of the most important sources of contamination of freshwaters and coastal waters, is a complex mixture of organic and inorganic chemicals. Degradation of chemicals during the treatment of sewage results in a wide range of products, some of which are transient, some more persistent, and many of which are not identified. A random screen of 20 organic man-made chemicals present in liquid effluents revealed that half appeared able to interact with the estradiol receptor *in vitro* (Jobling *et al*. 1995). Alkylphenol derivatives, notably nonylphenol, are well known environmental estrogens that appear to cause disruption of the endocrine metabolism of invertebrates as well as vertebrates, and phthalates, derivatives of some plastics are also implicated. A number of metals and metallic compounds also elicit symptoms that are known, or suspected, to be the result of interference with hormonally controlled metabolic activities. The best known such compounds are the organotin antifouling agents, particularly tributyltin (TBT), but metals, notably cadmium, have also been shown to affect endocrine mediated processes in invertebrates.

Estrogenic activity of environmental chemicals has nearly always been discovered because an estrogenic effect, either *in vivo* or *in vitro*, has occurred upon exposure to the chemical. Many

chemicals that exhibit, or appear to exhibit, estrogenic activity are quite different in structure from natural estrogens so it is not possible presently to assess whether a compound is likely to be estrogenic based solely on a knowledge of its chemical structure (White *et al*.,1994).

It is also important to bear in mind that a number of compounds with known endocrine-disrupting activity in invertebrates have been deliberately introduced into the environment as pesticides.

Table 2 lists some of the chemicals that have been shown to elicit effects that are believed or suspected of being the result of interaction with the endocrine systems of various invertebrate taxa.

Table 2. A representative list of chemicals suspected of causing disruption to invertebrate endocrine function. Additional examples are discussed in the text.

Contaminant	Species/life stage	Major effects	Mechanism	Reference
Herbicides				
Atrazine	*Daphnia pulex* (Crustacean)	Reduced fecundity and growth	Unknown	Schober & Lampert (1977)
Atrazine	*Daphnia pulicaria*, Egg-bearing adults (Crustacean)	No effect on survival or fecundity; neonate sex ratio shifted towards males	Unknown	Dodson *et al*. (1999)
Simazine	*Daphnia pulex* (Crustacean)	Impairment of reproduction; reduction in molt frequency	Unknown	Fitzmayer *et al*. (1982)
Diuron	*Daphnia magna*, adults (Crustacean)	Reduced fecundity; arrested development of juveniles	No mechanism suggested	Kersting (1975)
Metals				
Cadmium	*Daphnia magna*, juveniles (Crustacean)	Elevated ecdysteroid levels	None suggested	Bodar *et al*. (1990)
Cadmium	*Daphnia magna* (Crustacean)	Delayed reproduction; increased brood size; reduced size of neonates	None suggested	Bodar *et al*. (1988)
Cadmium	*Mytilus edulis*, adults (Mollusc)	Inhibits gonadal follicle development; stimulates spawning frequency	No mechanism suggested	Kluytmans *et al*. (1988)
Cadmium	*Macrobrachium Kistnensis* (freshwater prawn) *Barytelphusa cunicularis* (crab)	Hyperglycaemia	Reduced synthesis of crustacean hyper-glycaemic hormone (CHH)	Nagabhushanam & Kulkarni (1981b); Machale *et al*. (1989) (cited by Fingerman *et al*., 1996)
Cadmium, mercury, lead	*Uca pugilator* (fiddler crab), adult	Retarded limb regeneration (Cd and Hg only)	Depression of metabolic rate suggested as cause	Weis (1976)
Cadmium, PCBs, Naphthalene	*Uca pugilator*	Abnormal colouration	Inhibition of black pigment dispersing hormone (BPDH) synthesis	Fingerman & Fingerman (1978); Hanumante *et al*. (1981); Staub & Fingerman (1984a,b) (Cited by Fingerman *et al*. 1996)
Cadmium	*Asterias rubens*, adult (Echinoderm)	Delayed germinal vesicle breakdown during induced spawning; abnormal development of fertilized oocytes; abnormal embryo development; reduced ovarian growth; reduced steroid levels and P450	Adverse effects on steroid levels impacting on reproductive processes	den Besten *et al*. (1989; 1991a,b)

		levels in pyloric caeca		
Cadmium	*Strongylocentrotus intermedius* (crustacean)	Low fertilization success; formation of anomalous sex cells; arrested embryogenesis	unknown	Khristoforova *et al*. (1984)
Cadmium, zinc	*Asterias rubens*, adult (Echinoderm)	Increased steroid metabolism; elevated testosterone and progesterone levels	Effects of metals directly on enzyme activity or as a cofactor?	Voogt *et al*. (1987)
Selenium	*Daphnia magna* (Crustacean)	Suppression of growth and reduced egg production	Unknown	Johnston (1987)
Tributyltin (TBT)	*Caradina rajadhari* (freshwater prawn), mature	Retardation of regenerative limb growth and moulting; increase in weight and calcium content of cast exuviae	Inhibition of chitinolytic enzymes / Ca resorption	Reddy *et al*. (1991, 1992); Nagabhushanam *et al*. (1990)
Tributyltin (TBT)	*Uca pugilator* (fiddler crab), adult	Retardation of limb regeneration and moulting; abnormalities in the regenerates	No specific mechanism suggested – interference with developmental processes	Weis *et al*. (1987)
Zinc	*Uca pugilator* (fiddler crab), adult	Retarded limb regeneration	Depression of metabolic rate suggested as cause	Weis (1980)
PCBs				
PCBs (Clophen A50)	*Asterias rubens* (Echinoderm)	Abnormal development of fertilized oocytes; abnormal embryo development; reduced steroid levels in pyloric caeca; elevated steroid levels in gonads; reduced P450 levels in pyloric caeca	Adverse effects on steroid levels impacting on reproductive processes	den Besten *et al*. (1989; 1991b)
PCB29, Aroclor 1242	*Daphnia magna*, neonates (Crustacean)	Increased time to complete four moults	Presumed to act as antagonists of endogenous ecdysteroids	Zou & Fingerman (1997b)

Alkylphenols, phthalates				
Nonylphenol	*Balanus amphitrite*, larval stage (Crustacean)	increased production of larval storage protein	May represent promotion of an estrogen-dependent process	Billinghurst *et al*. (1999)
Nonylphenol	*Chironomus tentans*, all life stages (Insect)	Reduced survival at high concentrations. No other effects	No apparent oestrogen-related effects	Kahl *et al*. (1997)
Nonylphenol	*Daphnia magna*, prenatal – adult (Crustacean)	Mortality of adults and offspring; reduced brood size; reduction in length	No mechanisms suggested. Nonylphenol is an estrogen mimic in vertebrates.	Comber *et al*. (1993)
Nonylphenol	*Daphnia galeata mendotae*, adult (Crustacean)	Reduction in fecundity; developmental abnormalities	No specific mechanism suggested	Shurin & Dodson (1997)
Nonylphenol	*Daphnia magna*, adult females (Crustacean)	Reduced fecundity	Disruption of steroid metabolism	Baldwin *et al*. (1997)
Lindane, 4-octylphenol	*Daphnia magna*, neonates (Crustacean)	No effect on moulting		Zou & Fingerman (1997a)
Diethyl phthalate (DEP)	*Daphnia magna*, neonates (Crustacean)	Increased time to complete four moults	Presumed to act as antagonists of endogenous ecdysteroids	Zou & Fingerman (1997b)
Phthalate esters (various)	*Chironomus riparius*, larvae – adult (Insect)	No effects on survival, development or emergence		Brown *et al*. (1996)
Phthalate esters	*Daphnia magna* (Crustacean)	No effects on survival, growth, fecundity	None suggested	Brown *et al*. (1998)
Natural and synthetic steroids				
Diethylstilbestrol (DES)	*Daphnia magna*, all stages, multi-generation (Crustacean)	Reduced moulting frequency; reduced fecundity; disrupted testosterone metabolism	Inhibition of steroid hydroxylase? Antagonsitc interaction with 20-OHecdysone?	Baldwin *et al*. (1995)
Diethylstilbestrol	*Daphnia magna*, adult (Crustacean)	Increased duration of intermoult period	Antagonistic competition for the endogenous moult hormone receptor	Zou & Fingerman (1997a)
Diethylstilbestrol (DES) 20-hydroxyecdysone (20HE)	*Tisbe battagliai* All stages (Crustacean, copepod)	Inhibition of naupliar survival ; reduction in reproductive output	No mechanisms proposed. May not be endocrine-mediated	Hutchinson *et al*. (1999a, b)
Estradiol-17β, estrone	*Sclerasterias mollis* Starfish, adult (Echinoderm)	Increased ovarian estrone and progesterone levels; increased pyloric caeca estrone levels; incorporation of protein into oocytes	Ovaries and pyloric caeca may be estrogen target organs	Barker & Xu (1993)

Estradiol-17β	*Asterias rubens*, adults (Echinoderm)	Increased estrone levels in ovaries; increased oocyte diameters	Suggested that there has been enhanced incorporation of material into the oocytes	Schoenmakers *et al*. (1981)
Insecticides				
Methoprene (JH analogue)	*Rhithropanopeus harrisii*, larval stages to adult (Crustacean)	Mortality; increased intermoult duration	Methoprene may function as a JH analogue in crustaceans	Celestial & McKenney (1994)
Methoprene	*Mysidopsis bahia* all life stages (Crustacean)	Mortality; reduced growth; delayed brood release; reduced brood size	Likely to be a JH-related endocrine effect	McKenney & Celestial (1996)
Methoprene	*Palaemonetes pugio*, larval stages (Crustacean)	Mortality; inhibition of larval development	Likely to be a JH-related endocrine effect	McKenney & Matthews (1990)
Dieldrin	*Daphnia pulex*, *Eurytemora affinis* neonate – adult (Crustacean)	Mortality; time to first reproduction; reduced fecundity; increased time to maturity; reduced number of broods	None suggested	Daniels & Allan (1981)
Endosulfan (organochlorine pesticide)	*Daphnia magna*, juvenile – adult (Crustacean)	Reduced growth; decreased survival; reduction in brood size and number of broods; delayed maturation	Unknown but site of action of the pesticide is the CNS	Fernandez-Casalderrey *et al*. (1993)
Endosulfan	*Daphnia magna*, adult (Crustacean)	Increased duration of intermoult period	Antagonistic competition for the endogenous moult hormone receptor	Zou & Fingerman (1997a)
Endosulfan, Diazinon (organophosphate)	*Daphnia magna* (Crustacean)	Reduced rates of filtration and ingestion	Unknown but site of action of the pesticides is the CNS	Fernandez-Casalderrey *et al.* (1994)
DDT (insecticide) MCPA (herbicide)	*Lymnaea stagnalis*, adult (Mollusc)	Reduced fecundity	No mechanism suggested	Woin & Bronmark (1992)
Pentachlorophenol (broad spectrum biocide)	*Daphnia magna*, all life stages *(Crustacean)*	Mortality; reduction in fecundity; reduced elimination of testosterone metabolites	Possibly alteration of the metabolic clearance of endogenous steroid hormones	Parks & LeBlanc (1996)
Pentachlorophenol	*Paracentrotus lividus*, sea urchin, developing eggs (Echinoderm)	Alteration in embryonic development and differentiation; reduced echinochrome synthesis	No mechanism identified	Ozretic & Krajnovic-Ozretic (1985)
Naphthalene	*Procambrus clarkii*, crayfish, adult	Suppression of ovarian growth	Possible inhibition of gonad stimulating hormone release	Sarojini *et al*. (1994)

Mixtures				
Tannery and paper pulp mill effluent	*Macromia cingulata* larvae (Insect)	Shortened time to first moult (tannery); arrested larval moulting (paper pulp)	Possible JH mimics within the effluents	Subramanian & Varadaraj (1993)
Industrial effluent containing metals	*Chironomus spp.* (Insect)	Higher level of mentum deformities in exposed larvae in both field and lab conditions	No mechanisms suggested	Dickman & Rygiel (1996)
Organic and inorganic pollutants	*Chironomus* gr. *Thummi*, larvae (Insect)	Prevalence of morphological deformities related to pollutants	No mechanisms suggested	De Bisthoven *et al*. (1995)
Possible exposure to pollutants	*Chironomus spp.* (Insect)	Deformed mothparts; heavily pigmented head capsules; unusually thick head capsule and body wall	No mechanisms suggested	Hamilton & Saether (1971)
Volatile organic substances from fractionated distillation of crude oil	*Platynereis dumerilii, Nereis sucinea* (Annelid)	Induction of characteristic spawning behaviour	Interference with natural chemical signalling processes	Beckmann *et al*. (1995)
Sewage outfall	*Paramphiascella hyperborea, Stenhelia gibba, Halectinosoma spp.* (Mollusc)	Intersexuality	Not strongly correlated with proximity to outfall. Cause unknown.	Moore & Stevenson (1991, 1994)

4.1 Mollusca and tributyltin

There is a substantial amount of literature on the impacts of tributyltin on reproduction in Mollusca which remains the best, and possibly the only proven, example of endocrine disruption exerting effects at population level, rather than simply on the individual. Otherwise there is surprisingly little evidence of other chemicals giving rise to effects that are likely to involve endocrine disruption in molluscs. Cadmium produces impaired gonadal follicle development in mussels (Kluytmans *et al*., 1988) and Woin and Bronmark (1992) showed that exposure to non-lethal concentrations of DDT causes substantial reduction in the fecundity of the freshwater snail, *Lymnaea stagnalis*.

The following brief, and partly historical, account of the effects of TBT on aquatic organisms is derived partly from the recent review by Fent (1996) and the topic has also been critically reviewed recently by Matthiesen and Gibbs (1998).

Deleterious effects of organotin compounds were noted first in oyster (*Crassostrea gigas*) populations in Arcachon Bay, France, in the late 1970s and from 1977 to 1983 oyster production was severely affected (Alzieu, 1991). Shells developed abnormally and spatfall declined dramatically. Abnormally developed shells were also observed in the UK (Stephenson *et al*. 1991). Shell abnormalities included chambering and the formation of a protein-containing jelly

and effects on calcification occurred at concentrations as low as 2 ng l^{-1}, although sensitivity varies between oyster species. Larvae survived only a few days in Arcachon Bay water and slow growth and high mortality occurred in larvae exposed to 50 ng l^{-1} of TBT in laboratory tests. At a concentration of 20 ng l^{-1} growth of *C. gigas* spat was inhibited but the related species *C. virginica* was less sensitive. From a population point of view the effects on reproduction are much more important than shell deformities.

Important deleterious effects on reproduction in gastropods were also found about this time. Widespread incidence of abnormal development of reproductive organs in female *Ocenebra erinacea* (Neogastropoda) in France and in 1981 the imposition of male sex organs including a penis and vas deferens on mud snails (*Nassarius obsoletus* - Neogastropoda) was linked to TBT and termed imposex or pseudohermaphroditism. The frequency of imposex and degree of penis development was related to TBT levels and increased near harbours and marinas. Bryan *et al*. (1986) concluded that the effect is probably initiated at a sea water concentration of about 1 ng l^{-1} of tin. However, Smith and McVeagh (1991) found a high level of imposex in dogwhelks exposed to a nominal concentration of only 0.01 ng l^{-1}.

Gibbs *et al*. (1988) investigated the development of imposex in dogwhelks in relation to the degree of exposure to TBT at concentrations ranging from 1 to 100 ng l^{-1} of tin. All females developed intersex characteristics at all concentrations. Some females were sterilized through blockage of the oviduct at a concentration of 1 or 2 ng l^{-1} and at 3-5 ng l^{-1} virtually all were sterilized. One third of dog whelks reared in a concentration of 4 ng l^{-1} of tin in the laboratory were sterile as a result of blockage of the oviduct by the developing penis and vas deferens. At higher concentrations (10 ng l^{-1}) suppression of oogenesis also occurred, and spermatogenesis was initiated while at 20 ng l^{-1} some individuals developed a functional testis. TBT was shown to be by far the most effective inducer of imposex though limited effects were also produced by tetrabutyltin and tripropyltin (Bryan *et al*., 1988). Short *et al*. (1989) painted, *in situ*, the shells of *Nucella lima* with antifouling paint containing either tributyltin or copper. After one month significantly more of those painted with the TBT preparation had developed imposex than either the controls or those treated with the copper based paint. However, recently the specificity of the imposex phenomenon has been questioned since copper and other environmental stressors unexpectedly induced imposex in *Lepsiella vinosa* (Nias *et al*., 1993).

Imposex resembling that in *N. lapillus* has been induced in a variety of other species. Among prosobranchs, both mesogastropods and neogastropods, around 72 spp and 49 genera are believed to be affected worldwide. Susceptibility varies markedly between species with some showing effects at only the most severely polluted sites and in some species the imposex response causes little interference with reproductive activity. *Ocinebrina aciculata* (Gastropoda: Muricidae) has been shown to be more sensitive to TBT than even *N. lapillus* (Oehlmann *et al*., 1996) previously the most sensitive species known. Symptoms in the population, which is declining in France, include high numbers of sterilized females, male biased sex ratios, and poor reproductive performance and recruitment . Among the European prosobranchs for which TBT sterilization has been reported are *Nucella lapillus, Ocenebra erinacea, Hina reticulata, Trivia arctica* and *T. monacha* . Recently imposex has been reported in *Buccinium undatum* from the open North Sea where 20 years ago no signs of imposex were observed and this is apparently related to the intensity of commercial shipping traffic in this area (Hallers-Tjabbes *et al*., 1994).

Deposit feeding clam populations (*Scrobicularia*) are also reported to be negatively affected at TBT polluted sites (Ruiz *et al*. 1994a, 1994b, 1995a, 1995b). Embryonic development and burying behaviour of the clams are affected.

Whether imposex results in sterilization depends in part on the normal morphological configuration of the reproductive apparatus. Signs of imposex were observed in the majority of neogastropod taxa in waters of British Columbia frequented by boats and species could be divided into 4 types of anomalous morphological development, depending on whether the posterior end of the pallial vas deferens interferes, once formed, with the female genital pore located on the vulva (Bright and Ellis,1990).

Type 1	*Nucella lapillus*
	N. lamellosa
	Neptunea phoenecia
Type 2A	*Nucella caniculata*
Type 2B	*Nucella emarginata*
Type 3	*Searlesia dira*
	Ocenebra lurida
	Colus halli

The soft body morphology of *N. lamellosa* is virtually identical to that of *N lapillus* and the vas deferens was often seen to meet and overgrow the female genital pore indicating that, as in *N. lapillus*, imposex will lead to sterility and population decline in areas of high boating activity. Indeed a large proportion of females from the Victoria harbour breakwater were sterile and juveniles were under represented. On the inner surface of the breakwater only a few old sterile females could be found and on subsequent visits the snails became progressively difficult to find in this situation. *Neptunea phoenecia* showed similar characteristics but in this species actual retention of egg capsules was not confirmed.

Imposex does not result in sterility in *N. caniculata* because in this species the vulva elongates along with the penis and, although when fully formed, the vas deferens meets the base of the vulva, blockage was never observed. *Nucella emarginata* females respond in a similar way except that in this species the vulva enlarges as a thickening ring around the genital pore. In both species blockage of the genital pore is avoided by the enlargement of the vulva. No sign of sterility was observed in these species and they remained abundant.

In *Searlesia dira, Ocenebra lurida* and *Colus halli* there appears to be no potential for sterilization, even in extreme cases of imposex, because even when fully formed the male organs are well posterior to the vulva and so do not block the genital pore.
Thus, while it appears that the majority of neogastropods are susceptible to imposex, sterility due to blockage of the genital pore appears to be restricted to a few species.

Malformations of the genital tract in the periwinkle, *Littorina littorea,* a mesogastropod, were described for the first time in 1993 and related to TBT (Bauer *et al*. 1993, 1995) and Ronis and Mason (1996) found that exposure to TBT along with testosterone resulted in increased retention

of testosterone and its metabolites. These represent an intersex condition which is defined as a disturbance of the phenotypic sex determination between gonads and genital tract. This condition is distinct from that of imposex, in which male sex organs are superimposed on those of the female (Smith,1971). Female periwinkles develop either male features on the female organs or female sex organs are supplanted by the corresponding male formations. The result is a gradual transformation of the female pallial tract and can be described by a progressive scheme with 5 stages.

Normal females of *L. littorea* produce about 500 planktonic egg capsules, each containing 1-5 eggs, during a breeding season and free-swimming veliger larvae hatch after about 5 -6 days. Metamorphosis occurs after 4-7 weeks. Intersex development causes restrictions of the reproductive capability of females. In stage 1, loss of sperm is possible during copulation and so reproductive success may be reduced. In stages 2-4 females are sterile because oocytes and the capsular material leak into the mantle cavity (in stage 2), or the glands responsible for embryonic nourishment and/ or the formation of the egg capsules are missing (stages 3 and 4). Examination of intersex females have so far shown no sign of spermiogenesis or sex change.

The metabolism of TBT by marine organisms and links with observed effects was discussed by Lee (1991). In contrast to some other aquatic invertebrates that have been investigated (e.g. Lee, 1985, 1986, 1991; Rice, 1989) molluscs generally show a very limited ability to metabolize TBT. Lee (1985) found that oysters had metabolized only a very small amount of TBT after 6 days of exposure and Langston (1990) found that the clam, *Mya arenaria* also has a very limited ability to metabolize TBT.

Lee (1991) hypothesized that many of the observed effects in molluscs are related to enzymes involved in TBT metabolism. Female dogwhelks exposed to TBT showed an increase in testosterone levels and injection of testosterone, in the absence of TBT, also promotes penis development (Spooner and Goad, 1990). Imposex development is thus not a direct effect of TBT exposure but is mediated by steroid hormones. Hormones and toxicants share common metabolic pathways, notably the cytochrome P-450 system, that can give rise to interactions. Binding and inhibition of cytochrome P-450 enzymes by TBT may result in the production of testosterone (Spooner and Goad, 1990). Later research indicates that this occurs through inhibition of the cytochrome P450-dependent aromatase that is responsible for the conversion of testosterone to estradiol 17β (Spooner *et al*., 1991).

Since the cytochrome P-450 enzymes are also involved in synthesis of vitamin D, which regulates calcium metabolism, it is possible that the abnormal shell growth observed in oysters also results from interaction of TBT with cytochrome P-450 enzymes.

Recently Bettin *et al*. (1996) showed that TBT exposure at different concentrations induces a concentration and time dependent imposex development in female *Nucella lapillus* and *Hinia reticulata* and that similar and more rapid effects were produced by administering testosterone. Testosterone titres increased in a manner that was correlated with TBT concentration and duration of the experiment. Simultaneous exposure to TBT and the antiandrogen cyproterone acetate suppresses imposex development completely in *N lapillus* and strongly reduces imposex development in *H reticulata* proving that the effects are mediated by an increasing androgen

level and not directly by the organotin. Furthermore imposex development can be suppressed in both snails by adding estrogens to the aqueous medium, whereas artificial inhibition of the cytochrome P-450 dependent aromatase system using SH 489 as a steroidal aromatase inhibitor and flavone as a nonsteroidal aromatase inhibitor induces imposex development (Bettin *et al*., 1996).

In spite of the large amount of attention that has been given to the effects of tributyltin on marine molluscs there appear to have been no studies of the extent of the problem in freshwater. There may be an assumption that, with the ban on the use of tributyltin on small boats that applies in many countries, this is not likely to be an issue. Whereas the decrease in the seawater contamination in harbours as a result of regulation seems widespread this is not true of sediments. The degradation half life of TBT in marine sediment is in the order of years. Recent data on harbour and marine sediments indicate that residues remain at relevant levels, though very variable between marinas and estuaries.

The degree of contamination of freshwaters is less well known, particularly since regulation, though it is evident that levels fell after regulation.. In East Anglian rivers, levels of up to 0.032 $\Phi g\ l^{-1}$ were recorded in 1992, but in Lake Lucerne TBT concentrations were shown to be much greater in sediments by, a factor of nearly 1000 relative to the concentration in water (Fent, 1996).

4.2 Crustacea

Crustacean endocrine systems have been shown to be influenced by a range of chemicals, including vertebrate type steroids, metals, alkylphenols and pesticides.

4.2.1 Heavy metals

The effects of heavy metals on endocrine regulated processes in crustaceans have been reviewed by Fingerman *et al*. (1996, 1998) and include disruption of moulting, limb regeneration, blood glucose level, colour changes and reproduction.

Cadmium

Both cadmium (Bodar *et al*., 1990) and selenium (Schultz *et al*., 1980) have been found to have adverse effects on the process of moulting in Crustacea. Exposure of *Daphnia magna* to selenium delays the onset of proecdysis and in adults of this species exposed to cadmium the result is lengthening of the intermoult cycle, reduction in the number of successful ecdyses, reduction in body weight and reduced size of neonates.

Bodar *et al*. (1990) investigated the effects of exogenous ecdysteroids on moulting and reproduction of *D. magna* exposed to cadmium, in order to determine whether observed effects of cadmium exposure could be the result of changes in ecdysteroid titres. Cadmium produced a threefold increase in ecdysteroid level at an exposure concentration of 20 $\Phi g\ l^{-1}$. Administration of exogenous ecdysteroid also led to both a reduction in the number of successful moults and in the number of young produced but the size of the neonates was unaffected. As a result it was hypothesised that increased ecdysteroid levels in *Daphnia*, exposed to cadmium, were

responsible for the reduction in the number of successful moults but that the cadmium induced reduction in neonate size was not related to the increased ecdysteroid level. Ecdysteroid titres normally decline prior to moulting but when this reduction in ecdysteroids is overridden by the presence of exogenous ecdysteroids the animals do not moult. However, the authors also note that the essential-metal substitution characteristics of cadmium may provide an alternative explanation for impairment of moulting since several metallo-enzymes are involved in the moult process.

Heavy metals also adversely affect the hormonally regulated process of limb regeneration in decapods. Cadmium, mercury and lead have all been shown to retard limb regeneration and moulting in the fiddler crab, *Uca pugilator* (Weis, 1976). Cadmium and mercury exposure also results in a reduction in the number of tubercles on the regenerated limbs (Weis *et al*., 1986; cited in Fingerman *et al*., 1996)

Interaction between cadmium and methyl-mercury in respect of these effects is strongly influenced by salinity. In water with 30 ppt salinity, inhibition of limb regeneration and ecdysis in three species of fiddler crab, *U. pugilator , U. pugnax* and *U. minax* was much greater when exposed to a combination of methyl-mercury and cadmium than when exposed to either metal separately. However, at half this level of salinity the combination produces less inhibition of limb regeneration and moulting than does each metal separately. Zinc has a lesser effect than either methyl-mercury or cadmium but in combination with methylmercury the effects appear to be additive (Weis *et al*., 1992).

U. pugilator develops resistance to cadmium stress in respect of its effects on limb regeneration after exposure to 0.5 ppm for one week. However acclimatization does not influence the effects on moulting, indicating that previous exposure is protective in terms of the growth process alone and not through the neuroendocrine system which controls the moult cycle (Weis *et al*. 1992).

Blood glucose levels in decapods are regulated by the crustacean hyperglycaemic hormone (CHH). CHH is produced in the medulla terminalis X-organ-sinus gland complex in the eyestalk (see section 2.2). Exposure to cadmium gives rise to hyperglycaemia in the freshwater prawn *Macrobrachium kistnensis* (Nagabushanam and Kulkarni, 1981b). There is evidence, from research on another species of prawn *P. clarkii*, that this effect arises from interference by cadmium with CHH activity; stimulating release of CHH while at the same time inhibiting its synthesis. CHH activity in eyestalks of crayfish exposed to cadmium was less than in controls kept in clean water (Reddy *et al*. 1994).

Naphthalene and cadmium both induce hyperglycaemia in fiddler crabs but the effects differ from those cited above in that CHH activity in the eyestalks of crabs exposed to naphthalene was much more than in controls while CHH activity in eye-stalks of the crabs exposed to cadmium was reduced (Fingerman *et al*. 1996).

CHH also regulates amylase activity in the gastric juice of decapods by stimulating release of amylase from the hepatopancreas. In *P. clarkii* exposure to cadmium results in a significant increase in the pH of the gastric juice and a decrease in amylase activity again leading to the suggestion that cadmium simultaneously stimulates the release of CHH and inhibits the

synthesis of CHH (Reddy and Fingerman, 1994, cited in Fingerman *et al*. 1996).

In many crustaceans colour regulation is an important mechanism for avoiding predation. Colour change is regulated by pigment dispersing and pigment concentrating neurohormones that affect the distribution of the pigment within the chromatophores. Cadmium appears to inhibit the synthesis of the black pigment dispersing hormone (BPDH), since eyestalks of crabs kept in clean water contained more than three times as much BPDH as those of crabs exposed to cadmium (Reddy and Fingerman, 1995). Exposure to cadmium also results in diminished ability of *U. pugilator* to disperse black pigment. This effect is not only on the chromatophores but also on the neuroendocrine system which regulates these pigment cells. The polycyclic aromatic hydrocarbon, naphthalene, also affect the coloration of this species by inhibiting release of BPDH and, in contrast to the effect of cadmium, result in a substantial increase in the activity of BPDH in the eyestalks (Staub and Fingerman, 1984).

Tributyltin

Oberdorster *et al*. (1998) showed that although daphniids are highly susceptible to acute toxicity from tributyltin (TBT; 2.5 Φg l^{-1} of TBT was lethal to 60% of the organisms) exposure to lower concentrations resulted in no adverse effects on moulting or reproduction. Testosterone metabolism was enhanced in animals exposed to concentrations approaching lethal levels.

In contrast, TBT retards limb regeneration and delays ecdysis in fiddler crabs and produces anatomical abnormalities in the regenerates. There is curving in the wrong direction of the regenerated dactyl of the major chela of the males and the number of setae was reduced in limbs which regenerated in TBT-exposed animals (Weis *et al*., 1987). In the freshwater prawn, *Caradina rajadhari*, TBT exposure causes a dose dependent retardation of moulting (Reddy *et al*., 1992). TBT also inhibits calcium resorption from the exoskeleton of *C. rajadhari* and causes significant increases in the weight and calcium content of cast exuviae (Nagabhushanam *et al*., 1990).

Few aquatic invertebrates have been studied to determine if they can metabolize TBT but it is known that a number of decapods, including blue crabs (*Callinectes sapidus*) spider crabs (*Libinia emarginata*) and shrimp crabs (*Penaeus aztecus*) can metabolize TBT that enters via food or water (Lee, 1985, 1986; Rice, 1989). Stomach and hepatopancreas microsomes from crabs form hydroxylated derivatives of TBT via the cytochrome P-450 system (Lee, 1991). This is contrast to molluscs in which the ability to metabolize TBT appears to be very limited.(Lee, 1985; Langston, 1990)

4.2.2 Vertebrate-type steroids

No definitive role has been established for estrogens in crustaceans (see section 2.2.8) but experiments have identified the existence of physiological and biochemical functions that may be perturbed by environmental estrogens or other endocrine disruptors. These may be the consequence of true estrogenic effects (interaction with estrogen receptors) or alternatively estrogens and xenoestrogens may interact with other steroid hormone receptors or disrupt endogenous steroid metabolism through interactions with steroid metabolizing enzymes.

Baldwin *et al*. (1995) exposed *Daphnia magna* to the estrogen diethylstilbestrol (DES) which inhibited moulting in juveniles but not in adults. Juveniles moult more frequently than adults, which suggests that DES affects some factor that is responsible for frequent moulting in juveniles. It is possible that it competes antagonistically with the native hormone for the 20-hydroxyecdysone receptor and thus prevents the stimulation of moulting in the juvenile daphnids. Alternatively adults may be insensitive because they are better able to inactivate DES. DES was also found to reduce fecundity in second generation daphnids and to alter the rate of elimination of various testosterone derivatives. However, it should be noted that very high concentrations of DES, in environmental terms, were used in these studies (up to 0.5 mg l^{-1}).

4.2.3 Alkylphenols and phthalates

Pentachlorophenol inhibits both glucose and sulphate conjugation of steroids in invertebrates. Both processes are important elements of the steroid elimination/inactivation process in invertebrates. Baldwin *et al*. (1997) postulated that, because of its phenolic structure, 4-nonylphenol might have a similar inhibitory effect, giving rise to the disruption of steroid hormone homeostasis by elevating hormone levels, with the possibility that this would result in toxicity to steroid hormone-dependent processes such as reproduction.

These authors assessed the effects of 4-nonylphenol on the metabolic elimination of testosterone in daphniids. The animals were kept in water together with both 4-nonylphenol and radio-labelled testosterone. Exposure to nonylphenol increased the accumulation of exogenous testosterone, supporting the hypothesis that nonylphenol inhibits the metabolic elimination of the testosterone.

Whether testosterone is a physiologically relevant steroid hormone in crustaceans is not resolved (see section 2.2.8 for further details). Baldwin *et al*. report that exogenous testosterone has been shown to stimulate the conversion of ovaries to testes in ocypod crabs (Sarojini, 1963) and to initiate hypertrophy and hyperplasia of the androgenic gland, increase testis size and mobilize glycogen from the midgut to the testis in the paneid crustacean *Parapenaeopsis hardwickii* (Nagabhushanam and Kulkarni, 1981a). Testosterone has also been detected in the testes and serum of the American lobster (Burns *et al*., 1984) and in the ovaries of *Nephrops norvegicus* (Fairs *et al*., 1989). However a specific testosterone receptor has yet to be identified in crustacean tissues and therefore it is possible that androgens have no direct role within the crustacean endocrine system, or may serve as non-functional precursors of other steroid hormones. The authors suggest that the presence of androgens and enzymes capable of metabolizing these in crustaceans suggests that the effects elicited by 4-nonylphenol, are of physiological relevance. It is also possible that the glucosyltransferase and sulphotransferase enzymes responsible for the conjugation of testosterone also function with ecdysteroids, in which case 4-nonylphenol may also suppress the metabolic elimination of ecdysteroid conjugates (Baldwin *et al*., 1997).

Dodson and Hanazato (1995) speculated that the flexible sex ratio of cladocerans might be more easily influenced by hormone-like xenobiotics than that of obligate sexual species and predicted that the maximum *Daphnia* sex ratio observed during a year will be higher before 1945 than after. In support of this they cite data from Lake Mendota for 1895, 1975 and 1991 which show a dramatic decrease in the maximum frequency of males for two *Daphnia* species with no change

in a third, in which the frequency of males was low in all samples.

Subsequently Shurin and Dodson (1997) investigated the effects of nonylphenol and a toxic cyanobacterium in sex determination and development in *Daphnia*. In common with many cladocerans, female *Daphnia* divide their reproductive effort among females, males and resting eggs according to environmental conditions. Exposure of females to both nonylphenol and the toxic strain of *Microcystis* resulted in a change in fecundities in terms of the 3 types of offspring. Production of females and of resting eggs were affected in both experiments. Production of males was less sensitive to modification. Exposure to nonylphenol also produced a characteristic developmental abnormality at environmentally relevant concentrations. Prenatal exposure to nonylphenol gave rise to morphological abnormalities in a proportion of the juvenile *Daphnia,* at concentrations of nonylphenol within the range commonly found in waters receiving sewage effluent and well below the no effect level for acute and chronic effects on growth and reproduction in *Daphnia magna* of 0.024 mg l^{-1} (Comber *et al*., 1993). Only animals exposed prenatally developed these deformities which involved development of tail spines and swimming setae.

The effects of body size, life stage and sex on the toxicity of various alkylphenols to *Daphnia magna* were recently investigated by Gerritsen (1997; Gerritsen *et al*., 1998). Young and adults were similarly sensitive to the test and differences in rates at which survival was affected could be explained on the basis of differences in body size. Young, juveniles and adults were exposed to six alkylphenols and mobility, survival, growth and reproduction were monitored. Alkylphenols appeared to affect survival in two ways; via a direct (toxic) effect on survival and via an indirect effect resulting from immobilization.

Some of the females that survived showed conspicuous malformations of the carapace and based on the characteristic morphological differences between males and females it appeared that these had undergone an external masculinization. The occurrence of malformed females was linked to exposure concentration and the age at exposure. Malformations occurred together with an inhibition of growth and a stimulation of reproduction. The latter may be interpreted as an energy shift from growth to reproduction.

Growth and reproduction were both significantly delayed by exposure to alkylphenol and under certain conditions reproduction was stimulated by alkylphenols. Since alkylphenols simultaneously inhibited growth, stimulated reproduction and induced malformations it appeared that apart from its non specific mode of action as a polar narcotic the alkylphenol also interfered with the animal's hormone metabolism.

In contrast to the effect on the numbers of female offspring produced by *Daphnia magna* exposed to nonylphenol (Shurin and Dodson, 1997), Zou and Fingerman (1997) found that neither the synthetic estrogen diethylstilbestrol nor the estrogenic pesticide endosulfan affected sex differentiation in *Daphnia magna* although they both inhibited moulting. It is possible that structural similarities between hormonally active xenobiotics and steroid hormones such as estrogen are the basis for the hormonal activity of these chemicals in *Daphnia*. These similarities may enable the mimics to bind to ecdysteroid receptors and then act as antagonists rather than agonists of the ecdysteroids, presumably blocking the receptors for extended periods and

preventing endogenous steroid moulting hormones from binding to and turning on the receptor. However it is also possible that this effect is a more general response to stressors not directly involving ecdysteroids and their receptors.

Phthalate ester plasticisers are reportedly weakly estrogenic in vertebrates (Jobling *et al*., 1995). However, at a nominal concentrations of 1 mg l^{-1} no adverse effects were detected on the reproductive performance of *Daphnia magna* (Brown *et al*., 1998).

The settlement of the barnacle *B. amphitrite h*as been shown to be inhibited by exposure to environmentally realistic levels of 4-nonylphenol (Billinghurst *et al*., 1999). However the mechanism by which this occurred was not attributed to endocrine disruption. Exposure of nauplius larvae of the same species to the same levels of 4-nonylphenol caused an increase in the production of a larval storage protein (cyprid major protein; Shimizu *et al*., 1996) which has significant similarities to vitellin in the adult (Billinghurst *et al*., 1999). Exposure of the amphipod *Corophium volutator* to 4-nonylphenol enhanced fertility, reduced growth and caused a significant increase in length of antennae of the exposed males (Brown *et al*., in prep).

4.2.4 Pesticides

The use of synthetic mimics of juvenile hormones as insecticides introduced the problem of insecticides causing endocrine disruption in marine and fresh water crustaceans (Gomez *et al*., 1973). One such pesticide is methoprene. At a concentration of 1 mg l^{-1}, this chemical completely inhibits larval development in the estuarine shrimp, *Palaemonetes pugio* (McKenney and Matthews,1990). The first two larval stages and the final pre-metamorphic larval stage were more sensitive than intermediate larval stages. A similar study on the mud crab *Rhithropanopeus harrisii* indicated that this species was more sensitive to the single isomer formulation of methoprene than the double isomer formulation employed in the previous study, the duration of both survival and developmental being affected by exposure to 100 Φg methoprene l^{-1} (Celestial and McKenney, 1994).

Numerous aquatic toxicological studies have investigated the effects of another insect growth inhibitor, Diflubenzuron (DFB) on decapods and other crustaceans (reviews by Cunningham 1986 and Fischer and Hall. 1992). DFB affects reproduction, development and behaviour in crustaceans (Cunningham 1986). Significant effects are found at concentrations less than 10 Φg l^{-1} in most cases. However, diflubenzuron specifically inhibits chitin synthetase and may therefore not be considered strictly as operating via an endocrine pathway.

Exposure of *Daphnia* to the estrogenic pesticide endosulfan resulted in a range of sublethal effects including reduced growth, decreased survival and mean total young per female, an increase in the time to first reproduction, and a reduction in the mean number of broods (Fernandez-Casalderrey *et* al., 1993). Feeding behaviour of *Daphnia magna* is modified by exposure to the pesticides endosulfan and the organophosphorus insecticide diazinon (Fernandez-Casalderrey *et* al., 1994). The authors suggest such effects are mediated by the action of the pesticide in the central nervous system.

Various organochlorine pesticides are known to influence endocrine processes in vertebrates and effects that are suggestive of endocrine disturbance, at concentrations considerably below those

at which survival is affected, have also been demonstrated in some crustaceans. At a concentration of only 0.03Φg l^{-1} endrin delayed the onset of spawning and reduced the production of viable embryos in the shrimp *Palaeomenetes pugio* (Tyler-Schroeder, 1979). Low concentrations of toxaphene were shown to reduce fecundity in *D. magna* and dieldrin delays and reduces the number of viable offspring produced by the copepod, *Eurytemora affinis* (Daniels and Allan, 1981).

Schober and Lampert (1977) showed that atrazine has a marked effect on fecundity and growth in *Daphnia pulex* and *D. magna* at concentrations considerably below those that affect survival and Macek *et al*. (1976) found that the same herbicide impaired reproduction and development in the malacostracan, *Gammarus fasciatus*. Recently, Dodson *et al.* (1999) have reported that exposure of *Daphnia* to atrazine during embryogenesis shifts the sex ratio in favour of males. The authors suggest that this effect is more sensitive than those on survivorship and fecundity. In addition to negative effects on reproductive performance like those of atrazine, another triazine herbicide, simazine also gives rise to reduced frequency of moulting in *D. pulex.*

The urea herbicide, Diuron also affects reproduction in *Daphnia* (Kersting, 1975). The mechanisms underlying these effects are not known but they are strongly suggestive of endocrine disruption.

Carbamate pesticides have been shown to induce helmet formation in *Daphnia ambigua* but this is probably not an endocrine disruption process. Helmet formation also occurs in response to the presence of substances released by predators (e.g. *Chaoborus*) known as kairomones, and the pesticides successful in inducing this response are suggested to act directly via the nervous system (Hanazato, 1991; Hanazato and Dodson, 1995). Carbaryl and kairomone together interact to produce further synergistic reductions in body size and growth rate. However other stress factors, including pH may have a similar synergistic interaction with the natural and anthropogenic chemicals.

Piperonyl butoxide, is a synergist used in combination with pyrethroids, which acts by inhibiting the P450 catalysed metabolism of the pesticideIt has been shown that piperonyl butoxide inhibits production of viable offspring in daphniids while having no effect on survival, growth or reproduction (Baldwin and LeBlanc, 1994; LeBlanc and McLachlan, 1996; LeBlanc *et al*., 1995).

Kelthane (Dicofol) is a non-systemic acaricide with little insecticidal activity. According to Worthing and Hance (1991), impairment of reproduction in some birds by kelthane is due to DDT type contaminants and fish reproduction is also affected. Rao *et al*. (1992) investigated the toxicity of this pesticide in relation to the function of the neuroendocrine system situated in the eyestalks of the prawn, *Metapenaeus monoceros*. This system is involved in moulting, growth and development and various other metabolic and physiological processes. The eyestalks of some prawns were removed while other prawns were injected with eyestalk extract. Kelthane was highly toxic to prawns with one of the eyestalks removed by comparison with both controls and prawns injected with the extract. It was concluded that the hormones produced in the glands of the eyestalk influence toxicity through interaction with the pesticide.

4.2.5 Incidence of intersexuality in field populations of Crustacea

A discovery of a high frequency of intersexuality in Harpacticoida in the vicinity of Edinburgh's long-sea sewage outfall was followed up with a more extensive survey (Moore, 1991; Moore and Stevenson, 1994). Intersexuality was found to be common in *Paramphiascella hyperborea* and *Stenhelia gibba* and was present in two species of *Halectinosoma*. Intersexuality was widespread along the Edinburgh coastline and was recorded up to 10 miles from the outfall but there was no evidence for correlation between frequency and distance from the discharge. Most intersexes displayed similar secondary sexual characters with the sexually dimorphic prosome appendages taking the female form, the 5th leg intermediate between male and female, and the 6th leg and urosome male in character.

Intersexuality is well known among Crustacea including decapods, amphipods, isopods and planktonic copepods, (e.g. Hastings, 1981; Smith, 1977) but it appears to be extremely rare in harpacticoids. It was reported earlier by the same authors from around Scotland but otherwise the only other records relate to a single individual of *Amphiascoides debilis* and 1 of *Archisenia sibirica*.

In calanoids and several other taxa, intersexuality has been linked to parasitism but intersexes have often been found to exhibit no trace of parasitic infection and it has been suggested that transient parasitic infection may elicit intersexuality in such cases, although other external environmental factors may be involved. No parasites have been observed in any of the intersexes that were examined in this study. The initial finding was that 93% of *P. hyperborea* were intersex within 100 m of the terminus of the long-sea outfall. The large size of the individuals and the feminine nature of the appendages that develop first suggest that most intersexes commence life as females and undergo a masculinizing influence.

There is no indication of adverse effects on the macrobenthos around the outfall and all meiobenthic samples had a high copepod diversity indicating little or no influence on community structure. Tributyltin was considered as a possible cause but laboratory experiments with TBT did not induce such effects.

Sangalang and Jones (1997) describe the occurence of intersex in populations of lobsters exposed to sewage effluent but no definitive link to a specific causal agent was established.

4.3 Insecta

4.3.1 Vertebrate-type steroids

Vertebrate type steroids occur widely in insects (see Section 2.1.5 for details). Bradbrook *et al*. (1990) report that androgens and estrogens have been detected in cockroach, tobacco hornworm. mealworm and, milkweed bug. Eleven androgens and progestagens have been identified in haemolymph of a fleshfly, *Sarcophaga*. Titres of vertebrate type steroids were over a comparable range to the ecdysteroid titres but at present there is no known hormonal role for vertebrate type steroids in insects. The only uses mentioned by Bradbrook *et al*. (1990) are in defensive secretions from the prothoracic glands of water beetles and the rectal glands of carrion beetles.

The possible hormonal function of vertebrate type steroids and their origin remains uncertain and contentious (see Section 2.1.5). There is evidence that they can be synthesised from cholesterol but another possibility is that they are sequestered from food sources - however there seems to be no obvious relationship between the levels in the insect and those in its food.

It is of interest to note that some of the compounds known to exhibit estrogenic activity in vertebrates apparently have no effect, even at relatively high doses in insects. Exposure, from first larval instar to emergence to plasticisers (phthalates), which are weakly estrogenic in vertebrates, had no detectable effect on development of the midge *Chironomus riparius* (Brown *et al*., 1996). West *et al*. (1997) found no effects on survival, growth or reproduction of C*hironomus tentans* exposed to the dioxin TCCD and no effect on pupation was observable when the mosquito, *Aedes aegyptii* was exposed to the same compound (Miller *et al*., 1973). Similarly Kahl *et al*. (1997) found that nonylphenol did not affect the growth, survival, emergence, sex ratio, fecundity or egg viability of *Chironomus tentans*.

4.3.2 Insect growth regulators

Increased understanding of the role of hormones in the regulation of a number of insect life processes has led to the introduction of these hormones and their analogues as pesticides for use as biochemical control agents. Of particular interest are those hormones involved in the regulation of developmental and growth processes. Several major types of insect growth regulators have been studied to date of which the most extensive group are juvenile hormone analogues, which act as endogenous juvenile hormone and disrupt insect larval development.

Susceptibility to these compounds may differ markedly even between closely related species. Applications of 1000 Φg l^{-1} to larval habitats of *Culex pipiens quinquefasciatus* completely arrested adult emergence for 5 days. Control of adult emergence of *C tritaeniorhynchus* was effective at only 100 Φg l^{-1} but this rate of application was ineffective against *C pipiens molestus* larvae.

Non-target, aquatic animals also exhibit a wide range of susceptibility to the larval growth inhibiting pesticides. Diflubenzuron inhibits chitin synthesis during moulting and therefore may not be strictly considered an endocrine disrupter. Hanson and Garton (1982) observed effects of this compound on the fauna of experimental streams. Most susceptible among the insects were mayflies and stoneflies which were affected at concentrations of only 1.0 Φg l^{-1} . Dipterans were affected at ten times this dose while Coleoptera apparently were not affected at any concentration. Harrahy *et al*. (1994) exposed mayflies and stoneflies to Diflubenzuron in water and on contaminated leaves. Mayflies were sensitive to contaminated water at the lowest experimental concentration of only 0.6 Φg l^{-1}. Stoneflies were less sensitive to contaminated water but moulting was disrupted in larvae fed on treated leaves for 24 days. Survival of larvae was significantly affected 60 days after the treatment was terminated.The authors conclude that early life stages should be used in future tests, since these often coincide with autumn leaf fall, when introduction of contaminated leaves to headwaters may have an adverse effect on headwater communities.

4.3.3 Herbicides

Some herbicides have also been shown to adversely affect endocrine mediated processes in insects. It is well established that different cytochrome P-450 isozymes are involved in the metabolic pathways leading to the synthesis of 20-hydroxyecdysone and juvenile hormone (Wilkinson, 1985). Many cytochrome P-450 inhibitors are known to influence hormone-dependent functions. The bipyridium herbicides diquat dibromide and paraquat dichloride, which react with cytochrome P-450 enzymes, have been assessed for effects on growth and development in *Neobellieria bullata* (Diptera) larvae. Diquat dibromide caused prolongation of the first instar stadium, disturbance of the first moult, and delay of pupation in wandering larvae (Darvas *et* al., 1990). Paraquat dichloride was less active. This type of reaction is also caused by some cytochrome P-450 inhibitors such as feranimol, nuarimol and piperonyl butoxide. Feranimol is used as a fungicide and decomposes rapidly in sunlight; nuarimol is a similar compound used as a fungicide on barley; piperonyl butoxide is used as a synergist for pyrethroids.

4.3.4 Phytosteroids

Some phytosteroids (brassinosteroids) are structurally similar to ecdysteroids in that they both contain the entire cholesterol skeleton with the complete side chain and are very soluble in water (Luu and Werner, 1996). 20-hydroxyecdysone has been isolated from plants and shown to be structurally identical with the insect moulting hormone but since 1966 more than 100 phytoecdysteroids have been identified and experiments have shown that many of these structures demonstrate hormonal activity in insects (Adler and Grebenok, 1996). Effluents from pulp and paper mills were shown to influence moulting in aquatic insects. For example, moulting was arrested in dragonfly larvae (*Macromia cingulata*) when exposed to paper and pulp mill effluent (Subramanian and Varadaraj, 1993) and exposure of the same species to tannery effluent resulted in a shortening of the time to first moult, indicating that components of the effluents interfere with ecdysteroid metabolism.

4.3.5 Alkylphenols and phthalates

No effects were observed on the development (numbers and sex of emerging adults) of the midge, *Chironomus riparius*, of exposure to di-2-ethylhexyl phthalate or di-isodecyl phthalate at up to 10g kg^{-1} of sediment (Brown *et al*., 1996).

Kahl *et al*. (1997) report on the effects of 4-nonylphenol exposure on *Chironomus tentans* during a full life-cycle test. Although survival at 20 days was affected by the highest concentration used (200 Φg l^{-1} nominal) no effects on larval growth, emergence success or pattern, sex ratio, fecundity or egg viability were observed.

4.3.6 Morphological deformities in Chironomidae

A relationship between deformities of the mouthparts and other head capsule features of chironomid larvae were first documented by Hamilton and Saether (1970) who postulated the cause to be environmental exposure to heavy metals and pesticides. More recently De Bisthoven *et al*. (1995) screened concentrations of 10 heavy metals and 40 organic xenobiotics in river sediments and matched against types of deformity in *Chironomus thummi* (group) larvae. Particular types of deformities were associated with the occurrence of heavy metals, phthalates and DDT. However, some of these effects occur only over a very narrow band of concentration and environmental conditions and departure from these conditions apparently resulted in a

reduction in some types of deformities, often with an accompanying increase in some other type. A combined laboratory and field study in the Niagara River watershed revealed a significant increase in chironomid mentum deformities in organisms exposed to heavy metals (Dickman and Rygiel, 1996).

4.3.7 Hormones and invertebrate behaviour

A variety of hormones are responsible for organizing invertebrate behaviour (Truman and Dominick, 1983) and disturbance to normal behaviour is potentially a useful and probably sensitive end-point for use in laboratory bioassay. They are considered briefly here because they are better known in the insects than in other groups of invertebrates. For example, application of 20-hydroxyecdysone to larvae of *Ephestia* and some other insects induces prepupal cocoon spinning behaviour. A well known example is the so-called wandering behaviour of the tobacco hornworm *Manduca sexta,* which precedes pupation. This behaviour lasts for about 20 hours and is correlated with the endocrine changes that accompany the start of metamorphosis, including the disappearance of juvenile hormone and the appearance of a small peak in ecdysteroids (Riddiford and Truman, 1978).

Among freshwater insects behavioural patterns associated with case building or net spinning in caddis are processes that may be regulated by hormones. The form of the nets produced by *Hydropsyche,* for example, are complex and species specific and larvae of this genus are known to construct atypical webs when exposed to certain pollutants, although the mechanism for this disruption to normal behaviour has not been established.

Pollutants may also influence insect behaviour through apparent interference with pheromones. Blackwell *et al*. (1993) found that a polluted water sample and the synthetic oviposition pheromone *erythro*-6-acetoxy-5-hexadecanolide significantly increased oviposition by gravid female *Culex quinquefasciatus* (Diptera) measured against a clean water sample. The active semiochemical constituents of the polluted sample have yet to be identified.

4.4 Echinodermata

Khristoforova *et al*. (1984) and Gnezdilova *et al*. (1985) reported that oogenesis in sea urchins *Strongylocentrotus intermedius* was affected after short term exposure to cadmium at levels of 100 Φg l^{-1} or higher. Voogt *et al*. (1987) found that short term exposure to cadmium or zinc in the early stages of gametogenesis resulted in effects on steroid metabolism in the gonads and pyloric caeca of the sea star (*Asterias rubens*) and reproductive abnormalities were also observed by den Besten *et al*. (1989) in *Asterias rubens* exposed to both cadmium and polychlorinated biphenyls (PCBs). Den Besten *et al*. (1991) also confirmed that both cadmium and PCBs influence steroid metabolism in *Asterias,* causing a reduction of progesterone and testosterone levels in the pyloric caeca. Furthermore, steroid metabolism of the sea star can be influenced by these chemicals at tissue concentrations that are comparable with those recorded in animals from a heavily contaminated estuary or fed on mussels from this situation (den Besten *et al*.,1991b).

Short term exposure of *Asterias rubens* to 200 Φg l^{-1} of cadmium caused a reduction in ovary growth but there was no effect on the testes. Long term exposure to 25 Φg l^{-1} caused a delay in

ovarian growth which was obvious after 5 months exposure but by the end of the reproductive cycle the difference had become smaller. As in the short term experiments the growth of testes was not affected and the cadmium level in male gonads was lower than in ovaries by a factor of 6 (Den Besten *et al*. 1991a). No mortality occurred during the experiments and feeding rates of the exposed animals were unaffected.

The scale of the effect was dependent on the time of exposure. Production of oocytes in the sea star involves transfer of vitellogenic material from the pyloric caeca to the ovaries and in the course of oogenesis an increase in the weight of the ovary is accompanied by a reduction in the weight of the pyloric caeca. The largest increase in ovary weight occurs between November and March and the maximum effects on gametogenesis occurred when females were exposed between December and January. Females exposed to cadmium during this period showed almost no ovarian growth and the weight of the pyloric caeca remained high. This suggests that the cadmium had a negative influence on the transfer of vitellogenic material (Den Besten *et al*. 1991a)

Voogt *et al*. (1991) assessed that there was considerable evidence to indicate that steroids play an important role in the early stages of gametogenesis. Cytochrome P-450 enzymes are of critical importance in certain steroid biosynthetic pathways and, as part of the mixed-function oxidase (MFO) system these enzymes are also involved in the metabolism of xenobiotic compounds. Interference between the processes of steroid metabolism and xenobiotic metabolism can occur.

4.5 Conclusions

A considerable body of evidence strongly suggests that many organic and inorganic chemicals found as contaminants in the aquatic environment can exert adverse effects upon invertebrate species via mechanisms which may relate to interference with normal endocrine regulatory processes. However, the majority of these reports concern laboratory studies. There are few examples of the detection of possible endocrine disruptive effects in natural populations and only a single well-documented example of an endocrine disrupting chemical (TBT) exerting adverse effects on invertebrates (molluscs) at the population level. Therefore, while it must be accepted as a strong possibility that endocrine disruptive effects on aquatic invertebrate populations are common there is an inadequate database to support this assertion. Attributing the adverse effects of many chemicals to endocrine disruption is hampered by the relatively incomplete and fragmentary understanding of the endocrine system within many invertebrate groups.

It must also be noted that the endocrine systems of invertebrate groups differ markedly from each other, and from that of vertebrates (see Chapter 2). Most of the chemicals tested to date for effects as endocrine disrupters in invertebrates are known disrupters in vertebrate systems. Some of these, such as nonylphenol and some phthalate esters, fail to show evidence of adverse effects in exposed invertebrates. This emphasises the fact that some known vertebrate endocrine disrupters may have effects in invertebrates, others may not, and there may be a range of chemicals which have no effect on vertebrate systems but interfere with invertebrates.

5. ENDOCRINE DISRUPTING CHEMICALS - RELEVANT LEGISLATION AND REGULATIONS TO PROTECT INVERTEBRATES

5.1 International moves to integrate activities and policy with regard to endocrine disruption

The US Environmental Protection Agency (EPA) sponsored a workshop in April 1995 - "*Research needs for the Risk assessment of Health and Environmental Effects of Endocrine disruptors*" (Kavlock *et al*., 1996). Key Research areas identified were: basic research into mechanisms of endocrine disrupters, animal and cellular models; understanding the biological effects of low dose exposure; identifying the critical windows of susceptibility across species and characterising the source of population heterogeneity in response.

The *Food Quality Act (1996)* requires the EPA to test all pesticide chemicals, including the inert ingredients of pesticides for endocrine-disrupting effects. The EPA can also require the testing of any other substance that may have an effect that is cumulative to an effect of a pesticide chemical. The *Safe Drinking Act Amendments (1996)* authorise the EPA to develop and implement a program to identify and regulate substances that may have effects on humans similar to those produced by naturally occuring oestrogen or other endocrine effects.

Both these laws give the EPA highly specific time limits - they require the Agency to develop screening and testing strategies within two years and implement them within three.

Development of an international research program on endocrine-disrupting chemicals headed the agenda of the "*Intergovernmental Forum on Chemical Safety*" (IFCS) in February 1997 in Ottawa. According to F. C. McEldowney, associate director of international activities at the chemical manufacturers association. this conference Awas the first major international meeting that clearly put endocrine disruption on the screen as a broad issue.≅

The U.S. EPA and the European Union announced a joint effort to avoid duplication of work on endocrine disruption. A suggestion to establish an IFCS *ad hoc* working group to co-ordinate global activities and raise public awareness of the inherent hazards associated with these chemicals was not accepted for two reasons : a lack of money and a lack of definition as to what the working group was supposed to do.

The UN body called the "*Interorganisational Program for the sound management of chemicals*" was asked to compile and harmonise definitions of endocrine disrupters and report to the IFCS.

5.2 UK and EC legislation and regulations

Chemicals with Hormone-like effects were, in the Minister Declaration from the North Sea Conference, included in the category of chemicals with toxic effects. Nonylphenols, nonylphenol ethoxylates and related substances are included in the short-term action programme

of the Esjberg declaration which states that North Sea countries, before the year 2000, take action to substitue these chemicals with less environmentally hazardous chemicals or predominantly non-environmentally hazardous chemicals.

The Oslo-Paris Commissions (OSPARCOM) and the EU Commission were requested to initiate investigations and evaluations to improve the understanding of the consequences of chemicals with hormone-like effects, to help make informed decisions about necessary interventions. Full risk assessments of five phthalates, including DEHP and DBP, bisphenol-a and nonylphenol were to be performed within the EU framework for existing chemicals by 1997.

The following UK Government and EEC legislation and regulations are relevant to the control of endocrine disrupting substances in aquatic environments.

Water Resources Act 1991,
Environmental Protection Act 1990
Water Industry Act 1991
Dangerous Substances Directive (76/464/EEC)
Surface Water Abstraction Directive (75/440/EEC).
Integrated Pollution Prevention and Control (96/61/EEC)
Surface Water Abstraction Directive (75/440/EEC)
Sludge Directive (86/278/EEC)
Urban Waste Water Treatment Directive 99/ /EEC
Sludge (Use in Agriculture) Regulations (1989)(SI 1263),
Environmental Protection (Prescribed Processes and Substances) Regulations 1991
Integrated pollution Control Part 1 Regulations
Waste Management License Regulations

Control of endocrine substances in aquatic environments is within the scope of several Acts aimed at controlling pollution generally. Chief among these are the *Water Resources Act 1991,* the *Environmental Protection Act 1990* and the *Water Industry Act 1991*. These incorporate specific provisions under EC Directives, notably the *Dangerous Substances Directive (76/464/EEC)* and the *Surface Water Abstraction Directive (75/440/EEC).*

Section 85 of the *Water Resources Act* makes it an offence to cause or knowingly permit any poisonous, noxious or polluting material (or any solid waste matter) to enter any controlled waters. Clearly substances with the ability to disrupt endocrine function, whether in invertebrates or vertebrates fall within this definition.

The *Environmental Protection Act 1990* is concerned with substances for which a more precautionary approach is required. This Act regulates releases from some of the larger and potentially more polluting industries with the principal objective of preventing or minimising the release of prescribed substances and rendering harmless any such substances which are released using Best Available Techniques Not Entailing Excessive Cost (BATNEEC). Harm is defined as harm to the health of any living organism or other interference with the ecological systems of which they form a part. (In the case of man it also includes offence to any of his senses or harm to his property). A second objective is to control the releases from industrial processes so as to

minimise impact on environmental media.

Prescribed substances in relation to this Act are described in Schedules 4, 5 and 6 of the *Environmental Protection (Prescribed Processes and Substances) Regulations 1991* which relates to releases to air, water and land. Several of the listed substances are known to have endocrine disrupting properties, including dioxins, DDT, lindane and tributyltin.

The EC *Dangerous Substances Directive* and related Directives identify a number of chemicals as "List 1 substances" for which Environmental Quality Standards (EQS) or Statutory Water Quality Objectives (SWQO) are in place. Among the List 1 substances are several with known estrogenic activity, including DDT, HCH and dieldrin. The UK government made additional Statutory EQSs for 32 substances designated under List II in April 1998. These Regulations include endosulfan, atrazine and simazine all of which are known to have endocrine disrupting properties.

The EC Directive on *Integrated Pollution Prevention and Control (96/61/EC)* extends the approach of the *Environmental Protection Act 1990* to a wider range of industrial processes, and most significantly to agricultural practices and smaller industrial plants. Under Annex 3 of the Directive specific reference is made to the control of substances and preparations which have been proved to possess carcinogenic or mutagenic properties or properties which may affect reproduction in or via the aquatic environment. Thus there is provision here for control of other endocrine disrupting substances which affect reproduction in any aquatic organisms.

The *Surface Water Abstraction Directive (75/440/EEC)* sets environmental standards for the abstraction of drinking water and includes standards for several substances known to have endocrine disrupting properties. It aims to ensure that after treatment the water is fit for potable supply. Thus, although it is directed at human health rather than wildlife protection it is relevant in the present context since it involves additional controls over the quality of source waters.

The *Water Industry Act 1991* requires the sewerage undertaker to consult with the Agency and the Department of the Environment for the discharge of a prescribed substance into a sewer. The Agency does not necessarily have to be consulted over consents for other trade effluents to sewer but the sewage undertaker is required by law (*Water Resources Act 1991*) to notify the Agency of changes to the discharge of a "Dangerous Substance"

Disposal of sludge (i.e residual sludge from sewage plants) is controlled by the *Sludge Directive (86/278/EEC)* and *Sludge (Use in Agriculture) Regulations (1989)(SI 1263)*, supported by three Codes of Practice. Disposal of sludge at sea will cease as a result of the *Urban Waste Water Treatment Directive 99/ /EEC* . Alternatives methods of disposal are use as landfill or incineration or "recovery" (e.g. land-spreading,) depending on the source and chemical nature of the sludge. Incineration is controlled by the *Integrated Pollution Control Part 1* regulations which are enforced by the Agency or Local Authority in respect of air emissions and by the Agency in respect of disposal to land and water under the *Waste Management License Regulations* and *Water Resources Act (1991)*. Clearly disposal of sludge on land involves the possibility of contamination of freshwater with pollutants including endocrine disrupting substances and the amount of sludge being disposed of in this way is increasing, and likely to go

on doing so as a result of the increased levels of treatment requires by the *Urban Waste Water Directive.* Under the *Sludge (Use in Agriculture) Regulations* sewage sludge applied to agricultural land is not considered to be a controlled waste. The levels of metals (copper, cadmium, nickel, zinc, lead, chromium) in the sludge and in the land determine whether the sludge can be disposed of in this way but organic contaminants are not measured. Sludge applied to non agricultural land is treated as controlled waste unless the application results in ecological improvement and metal levels are not exceeded. In this case a Waste Management Licence is required.

In addition to its responsibilities in respect of the control and monitoring of pollution in the aquatic environment the Agency also can advise Government that the use of certain substances should be controlled or banned, in cases where the substance in question represents a serious threat to the environment (or human health). The Government has the ability to control such substances through the *Control of Pesticides Regulations 1986 (SI1510)* or through the *Environmental Protection Act 1990*. The Agency supports control at source as the most powerful and appropriate means of reducing environmental exposure to harmful substances but for this to be effective requires that hazardous substances are identified through sound testing regimes. At present regimes for testing substances for the chronic effects of endocrine disruption that may result from low exposure levels have not been adequately developed.

5.3 Biological monitoring in the UK

The Environment Agency has a national strategy for monitoring inland and coastal waters that is currently under review. A probable outcome of the review is that there will be increased emphasis on the importance of biological monitoring and, by implication, less reliance on chemical data alone.

The programme of biological monitoring is based on the RIVPACS model and associated methodologies (e.g. Murray-Bligh *et al.* 1997). The core strategy for rivers involves a quinquennial survey of 7000 sites, together with a corresponding number and distribution of chemical survey sites. At present the strategy for surveillance of lakes is under development but, in respect of ponds, the "Pond Action Framework Directive" requires monitoring of fish, macroinvertebrates and plankton. In future, surveillance of all aquatic habitats, including marine, will be based to a larger extent on ecological considerations rather than the emphasis being largely on chemistry. In our view this is a positive and sensible step because of:- (a) the impossibility of analysing water, let alone sediments for the vast array of potential pollutants that may be present; (b) the problems of testing chemicals for biological activity, especially in respect of potential sublethal effects at low concentrations; (c) the largely unresolved questions of additive or synergistic effects of mixtures of pollutants.

Apart from the quinquennial surveys, which are undertaken in all regions, the amount of effort expended on biological monitoring varies between regions. Those with predominantly stressed environments carry out more routine monitoring than those with largely clean conditions, but all regions carry out operational monitoring to follow up pollution events and to identify sources of pollution. There is some variation in procedures and methods but in respect of invertebrate monitoring these are broadly based on the RIVPACS approach.

Biological water quality monitoring using invertebrates is based on community structure but does not assess the condition of individual organisms, (e.g. incidence of deformities) or populations (e.g. abnormal sex ratios or unusually high incidence of intersex or imposex). Therefore the current invertebrate samples derived from the monitoring programme will require increased analysis in order to generate the data needed, for example the incidence of deformities or sex specific markers.

5.4 Conclusions

Considerable legislative and regulatory mechanisms are in place or under discussion in both the US and Europe concerning the classes of chemicals demonstrated to possess, or suspected of possessing, endocrine disrupting activity in vertebrates. With regard to aquatic invertebrates, the primary constraint on further action is the absence of systematic evidence of adverse effects of endocrine disrupting chemicals on natural populations. Until such evidence is available, or it is demonstrated that invertebrate populations are not experiencing widespread interference by endocrine disrupting chemicals, further specific legislative action targeted towards the protection of invertebrates, will not be supportable.

Further reference
http://www.epa.gov - search Aendocrine≅ is a good source of policies established in the USA

6. GAPS IN KNOWLEDGE AND RESEARCH PRIORITIES

6.1 Is there a need for research?

Chemicals that cause endocrine disruption, or appear to do so, in invertebrates (and vertebrates) are diverse and frequently do not structurally resemble the hormones which they appear to mimic or interfere with. It is therefore not feasible to predict with any level of confidence whether particular chemicals or groups of chemicals will, or will not exhibit endocrine-related activity in particular invertebrate taxa. With the exception of TBT-disrupted reproductive function in molluscs, discovered as a result of field observations in the first instance, there are few documented case of endocrine disruption in invertebrates. The example of TBT remains the only chemical "case-study" where ecologically-significant population level effects of endocrine disruptors have been found. Even the reportedly high incidence of intersex in natural populations of harpacticoids in the North Sea has had no obvious impact on population size or community structure.

It is of course possible that the lack of evidence is the result of a failure to look for signs of endocrine disturbance in invertebrates, in both freshwater and marine ecosystems. Surprisingly few studies have been initiated to date, especially in view of the enormous importance of invertebrates with regard to ecosystem structure and function. It is worth remembering that invertebrates constitute 95% of all animal species. Recent reports in the scientific literature have provided preliminary evidence of changes in appendage morphology, the induction of vitellogenin, alterations in larval settlement, the development of ovotestes, and possibly an increased frquency of intersex in different crustaceans exposed to putative endocrine disruptors. Other invertebrate groups have simply not been investigated to date.

Areas which may be considered priorities for further work are considered below.

6.2 Basic endocrinology

One factor which has facilitated study and understanding of the phenomenon of endocrine disruption in vertebrates is the fact that all vertebrates, from fishes through to primates, share a common endocrine system, albeit with some species specific variation; for example, gonadal steroids in all vertebrates share closely related (in some cases identical) structures and roles. In contrast, an understanding of endocrine disruption in invertebrates requires that the considerable diversity of endocrine systems between the major invertebrate phyla is somehow accomodated.

As is clear from the brief overview of our state of knowledge regarding the endocrinology of invertebrates provided in Chapter 2 the extent of our knowledge varies greatly according to the taxa. 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. Clearly, an understanding of the endocrinology of relevant organisms is important if the mechanisms by which environmental contaminants elicit effects are to be accurately attributed.

However, 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 (see Chapter 3 for discussion). Therefore, while undoubtedly important, research into the basic endocrinology of invertebrate species need not be considered a priority requirement. Existing research effort, which is driven by factors other than concerns arising from consideration of endocrine disruption, should be sustained and will continue to assist understanding within the field of invertebrate endocrine disruption.

6.3 State of invertebrates in the aquatic environment

With regard to the natural environment, 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. To do this effectively, it will be necessary to identify a range of "sentinel" organisms.

6.3.1 Selection of species

It is important to emphasise that several sentinel species will be required since the endocrinology of the invertbrates differs widely among the phyla. Thus, for example, one cannot expect a Asentinel crustacean≅ to provide early warning of endocrine disruption in echinoderms or annelids; representatives of each phylum will be required. The following criteria for the selection of sentinels are based in part on discussions held at the Institute for Environmental Health workshop on "The Ecological Significance of Endocrine Disruption" held in Leicester in July, 1997. Sentinels should :-

- be common and widespread
- reproduce sexually and be sexually dimorphic
- be relatively insensitive to "conventional" pollutants
- have a well understood biology
- have a reasonably well understood endocrine system

In combination they should be representative of:-

- a range of life styles and feeding habits
- a range of endocrine "types"

With regard to assessing the endocrine disrupting potential of new and current environmental chemicals in relation to invertebrates, there is an urgent need to develop a broad range of bioassays. These might utilise many of the sentinels organisms described above, but to faciliate use in laboratory bioassay species should in addition :-

- have fairly rapid generation times

! be readily cultured and amenable to experimentation

Although it is preferable for the same organisms to be used as sentinels and for bioassay it is unlikely that this will always be possible and potentially good sentinels should not be overlooked because they are not suitable for use in the laboratory. Similarly, bioassays should not be discounted if they utilise organisms that are not necessarily be typically found in the field situations where endocrine disruption occurs. The primary purpose of bioassays is to permit assessment of the relative risk of endocrine disruption associated with different chemicals and chemical mixtures. It is an added bonus if they provide ecologically relevant information.

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

Annelida
Little information is available concerning suitable annelids to use either as sentinels or bioassay species. In freshwater environments, leeches (Hirudinea) may be particularly relevant. In the marine environment, annelid species used in other types of toxicity testing may be valuable. The worms, *Platynereis dumerilii* and *Dinophilus* spp. may be suitable; the latter has already been used in preliminary studies of endocrine disruption (Price and Depledge, 1998).

Mollusca
Neogastropods, dogwhelks and periwinkles, are currently used as indicators, especially with respect to tributyltin contamination. Aberrations in the shell structure of bivalves (mussels, clams, oysters) are also likely to be useful indicators. In freshwater there is little if any indication that endocrine related impacts have been looked for among invertebrates. We recommend that gastropods and bivalves from a range of sites below sewage works and industrial effluents should be surveyed for evidence of reproductive or other morphological abnormalities. If effects are detected, further surveys should be carried out to establish whether there is also evidence of impacts at the population level.

Crustacea
Daphnia have been used extensively for chronic bioassay in laboratory testing of chemicals but their suitability for this purpose has been questioned on several grounds. In our view the most important criticism is that they are normally maintained in the laboratory under conditions that favour asexual reproduction, whereas it may well be the sexual phase that is more likely to be impacted upon by endocrine disruption. *Daphnia* are also unlikely to occur sufficiently commonly in riverine situations and are therefore not good candidates for sentinel organisms. Other Crustacea, such as *Asellus* and possibly *Gammarus* are likely to be more suited to this role. Both are widespread and sexually dimorphic and amenable to laboratory handling. *Asellus* is particularly robust in this context and is also likely to occur in reasonable abundance below treated sewage effluents.

Amphipoda and Isopoda should also be useful in respect of marine surveillance and Harpacticoida are also likely to be useful in view of the high incidence of intersex recorded in populations in the North Sea, apparently associated with a long sea sewage outfall. In all of these

cases (freshwater and marine) bias in sex ratios and evidence of intersex development are predicted to be useful as biomarkers but further research on the "normal" ranges of variation is also required to aid interpretation of resulting data. Occurrence of abnormalities in regenerated limbs or in other exoskeletal structures of amphipods and decapods may also be a useful biomarker in marine habitats.

Insects

Chironomidae have for some time been known to develop abnormally when exposed to certain pesticides and industrial effluents. It is possible that these are the result of disturbance of endocrine functions associated with development and moulting. Surveys of the incidence of such abnormalities in relation to sites where endocrine disrupting chemicals are likely to occur should be accompanied by laboratory studies to try to establish relationships between specific chemicals or types of chemical and characteristic deformities.

Endocrine disruption may also be expected to influence sex ratios and cause the development of intersex in chironomids, as happens in some cases of parasitic infestation. Sex ratio is most readily determined through examination of pupal exuviae but determination of intersex would require examination of the adult insects which could be collected conveniently and cheaply using light traps.

The most likely candidate as a sentinel chironomid in respect of each of these indicators is *Chironomus riparius* which is generally common in rivers where there are sewage effluents or a degree of pollution from industrial pollutants, at concentrations not tolerated by most chironomids or other insects.

Echinodermata

The literature indicates that both sea urchins and sea stars are likely to be useful subjects for bioassay. Attempts have yet to be made to assess endocrine disruption in echinoderms *in situ*. There is no reason why such an approach should not be successful.

6.3.2 Variables to be measured

Depledge and Billinghurst (1998) recently proposed a strategy for the assessment of endocrine disruption which incorporates the biomonitoring and bioassay approaches mentioned above, but in addition, recommends the use of cellular and biochemical biomarkers of endocrine disruption as well as detailed histopathological studies. Bioassay species with short generation times can be exposed to potential endocrine disruptor chemicals in the laboratory and parameters such as growth, reproductive output, viability of offspring, sex ratio and transgenerational effects can be assessed. If effects are observed, it is important to note that this is not necessarily evidence of endocrine disruption (for example, metabolic toxicity might account for such effects). Thus other evidence is required. With regard to vertebrates, the biomarker approach has proved to be helpful. Biomarkers are molecular, biochemical, cellular, physiological or behavioural responses that can be measured in tissue or body fluid samples, in excreta or at the level of whole organism, that provide evidence of exposure to, and/or adverse effects of pollutant chemicals. In fish, the induction of vitellogenin (egg yolk protein) in males has been used extensively to signal exposure to xenoestrogens. A similar approach is feasible using invertebrates. For example, vitellogenin can also be induced in male crabs by xenoestrogen exposure (Bjerregaard, personal

commnication) and vitellin (the precursor of vitellogenin) can be induced in barnacle larvae (Billinghurst *et al*., 1998). Once again, biomarkers do not conclusively show that endocrine disruption is the prime cause of the changes in Darwinian fitness parameters mentioned earlier, but they do add to the weight of evidence that endocrine disruption has occurred. Finally, Depledge and Billinghurst (1998), highlight the importance of histopathological evidence. Studies on lobsters, for example, provided clear evidence of the formation of ovotestes in animals exposed to sewage. Taking all of the evidence together provides a strong case that endocrine disruption has occurred.

6.4 Ecotoxicology – mechanisms and confirmation of cause and effect

It is known that adverse effects may occur via disruption of endocrine processes in invertebrate species either intentionally (e.g via pesticides) or unintentionally (e.g. TBT exposure). What is not yet known is the extent to which invertebrate populations are at risk to the adverse effects of chemicals acting via the endocrine system. A major issue which must be considered in determining research priorities is whether it is necessary to distinguish effects arising from endocrine-disrupting chemicals from effects arising from chemicals which operate via other mechanisms.

Many of the existing invertebrate toxicity testing protocols provide data on all the major processes which might be impacted by endocrine-disrupting chemicals, to a potentially greater extent than is the case for vertebrates. It is likely that thorough testing with existing invertebrate protocols would detect chemicals which exert adverse effects via the endocrine system. However, existing protocols tend not to provide data on the mechanistic basis of observed toxic effects.

What must be determined is whether it is appropriate to instigate a test regime (or continue with existing test strategies) which identifies effects without necessarily identifying the route by which those effects occur, or whether it is important to discriminate between compounds which act via the endocrine system and those which do not. 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. The most appropriate use of 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.

It is probably not appropriate to include studies of the mechanisms of endocrine disruption in routine environmental management programmes; nonetheless, they should be supported in universities and research institutes. An understanding of mechanisms may help to more accurately assess risks and shift patterns of chemical use to minimise future problems.

7. RECOMMENDATIONS

7.1 Endocrinology of invertebrates

- 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.

- An understanding of the endocrinology of relevant organisms is important if the mechanisms by which environmental contaminants elicit effects are to be accurately attributed.

- However, 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.

- Therefore, while undoubtedly important, research into the basic endocrinology of invertebrate species need not be considered a priority requirement in this context. Existing research effort, which is driven by factors other than concerns related to environmental endocrine disruption, should be sustained and will continue to assist understanding within the field of invertebrate endocrine disruption.

7.2 Monitoring of invertebrate 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.

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

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

- 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.

7.3 Ecotoxicology

- Many existing invertebrate toxicity testing protocols provide data on the major processes which might be impacted by endocrine-disrupting chemicals, to a greater extent than is the

case for vertebrates.

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

- What must be determined is whether it is appropriate to instigate a test regime (or continue with existing test strategies) which identifies effects without necessarily identifying the route by which those effects occur, or whether it is important to discriminate between compounds which act via the endocrine system and those which do not.

- 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.

- The most appropriate use of 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.

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