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Endocrine disruption horizon scanning: molecular and genomic contributions

Science Report – SC030276/SR2

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Steve Killeen

Head of Science

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

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

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

Executive summary

Endocrine disruption (ED) is an issue that has been around for well over a decade, has given rise to public concern worldwide and is dominant in ecotoxicology. This review is part of a series on endocrine disrupting chemicals (EDCs), which are thought to cause hormonal and sex changes in freshwater organisms such as dogwhelks and fish. It specifically focusses on the growing use of genomics and how it has improved our understanding of the effects of chemicals on wildlife.

Most studies of endocrine disrupting chemicals have focused on high doses or very polluted environments. However, with the advent of genomic technologies we can now measure effects at much lower doses, to establish whether important changes are occurring at the level of gene expression (gene activation or suppression).

Genomics encompasses several disciplines including transcriptomics, the study of gene expression at the transcript level. DNA chips and microarrays now enable the study of thousands of genes simultaneously. Proteomics is the study of a protein's specific function, how it interacts with other proteins, and protein differences between healthy and diseased cells. Metabolomics is the newest genomic technology, but because of its vast potential is moving at a very fast pace. Metabolomics is a functional readout of the physiological state of the cell at any point in time and is therefore a measure of an organism’s phenotype. Probably the greatest advantage of these techniques is that they can reveal the mechanism of action of a toxicant. Understanding the mechanisms of action of EDCs is critical, as this is central to determining whether the chemical is an endocrine disrupter and is causing an adverse response in an individual or population.

Genomic tools have already been used to study the effects of EDCs on model organisms, mainly to assess gene expression responses to exposure to EDCs (especially in fish). These transcript studies have enabled researchers to identify and functionally characterise genes involved in endocrine disruption, and work towards developing biomarkers. They have also been used to clone steroid and non-steroid receptors in wildlife, to study the function of hormone receptors and gain insights into underlying mechanisms. Proteomic and metabolomic approaches have been less forthcoming, although they are on the increase.

While these tools are available for studying changes in gene transcripts, proteins and metabolites, the techniques are at different stages of development and as a result, their potential integration into regulation will occur at different points in time. In terms of applying genomic tools in ecotoxicological testing, at present they are not in a state to replace traditional methods, but can nevertheless enhance the type of information derived from an experiment.

This report concludes that further research is needed to establish and validate the use of genomics for environmental monitoring, before these tools can be considered for regulatory application.

Research is needed to:

- validate the tools being developed;
- develop datasets for various species and environments, documenting normal gene expression profiles and fingerprints of exposure;
- further develop resources for analysing the datasets generated, and maintain publicly available databases to support work in this area of research;

- develop scientific partnerships of experts in ecotoxicology and genomics, in order to fully make use of the tools that these technologies are able to provide.

Among the most promising emerging technologies, microarrays and real-time PCR are likely to be especially useful for the detection, monitoring and mechanistic understanding of the risks posed by environmental chemicals. Microarrays can help elucidate the mechanisms of action of individual chemicals and identify new biomarkers for environmental monitoring, but their complexity and cost restricts their use to large specialised laboratories. Once molecular biomarkers are identified, real-time PCR can be applied to large numbers of samples at a relatively low cost, making this technique particularly suited for environmental monitoring.

The genome sequencing efforts of the last decade have opened new avenues for biologists in all areas of research. However, progress in the application of these resources to ecotoxicology will depend on progress in our understanding of the relationships between gene expression alterations and phenotypic changes as a result of chemical exposure, and this area requires further research.

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Contents

1 Introduction

A resolution was adopted in October 1998 by the European Parliament, calling upon the European Commission to take action on the issue of endocrine disruption (ED). A report published in March 1999 by the Scientific Committee for Toxicity, Ecotoxicity and the Environment (SCTEE) identified a 'potential global problem' for wildlife, and highlighted a possible link between reproductive and development effects resulting in changes in populations after exposure to suspected endocrine disrupting chemicals (EDCs). Following this, a *Strategy for endocrine disrupters - a range of substances suspected of interfering with the hormone systems of humans and wildlife* (COM, 1999) was published in December 1999 to address the ED issue. See http://ec.europa.eu/environment/endocrine/index_en.htm and http://ec.europa.eu/research/endocrine/activities_links_en.html for more detailed information and other useful links to ED information.

Several comprehensive reports on ED have been published (see Damstra *et al*., 2002; Botham *et al*., 2003; WHO/UNEP/ILO, 2004, with a summary of key research needs for ED). Therefore, this report does not provide a full review of ED *per se*. Instead, it offers examples of where genomics or the so-called 'omic' technologies and molecular biology have been applied to increase our understanding of ED in wildlife, or where there have been advances in genomics that could be applied to endocrine research. Global efforts are underway to understand how environmental chemicals, so-called EDCs, interfere with the normal functioning of the endocrine system in animals. In addition, there is a need to assess the biological consequences of such interactions at environmentally relevant concentrations, in order to establish safe levels of chemical exposure and ultimately protect both the natural ecosystem and the community.

This review aims to:

- Briefly introduce genomic technologies;
- provide a brief overview of ED in wildlife;
- present examples of research conducted on endocrine disruption using genomics and molecular approaches;
- summarise the main areas of omic technologies and highlight issues of importance to consider when using them in ecotoxicology.

1.1 Definition of endocrine disruption

Since February 2004, the US Environmental Protection Agency (US EPA) has defined an endocrine disruptor as "*an exogenous agent that interferes with the synthesis, secretion, transport, binding action or elimination of natural hormones in the body which are responsible for the maintenance of homeostasis, reproduction, development or behaviour*". It states that ED is not an "*adverse effect per se, but rather a mode or mechanism of action potentially leading to other outcomes, for example, carcinogenic, reproductive, or development effects, routinely considered in reaching regulatory decision*". An endocrine disrupting chemical has been defined by the Organisation of Economic and Cooperative Development (OECD) as "*an exogenous substance or mixture that alters the function(s) of the endocrine systems and consequently causes adverse health effects in an intact organism, or its progeny or (sub) populations*" (Lister and Van Der Kraak, 2001).

1.2 The endocrine disruption phenomenon

ED is an issue that has been around for well over a decade, has given rise to public concern worldwide and is dominant in ecotoxicology. Matthiessen (2003) states that effects seen in wildlife that can now be attributed to ED have been around since the 1940s and that it is a misconception to think that the study of ED began with the famous meeting at the Wingspread Conference Centre, Racine, Winsconsin in July 1991, organised by Theo Colborn and coworkers (their website *Our stolen future* http://www.ourstolenfuture.org/index.htm provides regular updates about cutting edge science on endocrine disruption). A study in the 1950s by Burlington and Lindemann found endocrine disrupting effects in white leghorn male chicks exposed to DDT. Male chicks had severely impaired development of secondary sexual characteristics. There was a reduction in the development of testes and in the colour and size of the combs and wattles. It was suggested that feminising effects associated with DDT exposure were due to an oestrogen-like effect.

Therefore, it is true to say that the study of ED has been around for decades, but the importance of such findings was only realised at the Wingspread Conference in 1991 when the concept of ED was initiated. The foremost observation upon which the concept was based was "*that some exogenous compounds of both natural and synthetic origin can interact with hormonal systems by one of several mechanisms, including mimicking or blocking receptor binding, altering the rates of hormonal synthesis or metabolism, or affecting receptor availability*" (Myers *et al*., 2001).

Observations of ED were visible in different species such as birds and fish, and they all had common characteristics which included:

- chemicals affecting embryos, foetuses and newborns in a different way to adults;
- effects not only in the parent exposed to the chemical, but also in its progeny;
- windows of sensitivity to chemicals, outside of which no effects may be observed;
- if exposed to a chemical in very early stages of life, an adverse effect may be delayed and not become visible until adulthood.

The endocrine disruption phenomenon is not only accepted by the scientific community but also by regulatory bodies. Regulators recognise that acceptable limits for environmental EDCs are required. The Environment Agency, UK, sets threshold limits for steroid oestrogens in sewage effluent. However, it is now realised that acceptable limits need to be set for both individual compounds and for mixtures of chemicals. In order to set acceptable limts of EDCs an understanding of the relationship between exposure and adverse effect are needed. Exposure tools that can help to improve our understanding of this relationship are essential but lacking. This knowledge gap is a limiting factor allowing the effective evaluation of risk posed by potential EDCs. New and emerging technologies such as 'omic' technologies have the potential to yield additional valuable information for understanding the mechanisms of action of EDCs and bridge that knowledge gap.

2 Omic technologies

Most studies assessing the effects of EDCs, whether laboratory or field-based, have focused on the use of high doses or very polluted environments together with traditional toxicological endpoint measurements. The advent of new 'omic' technologies which allow the measurement of effects at much lower doses suggest that EDCs in the microgram per litre and lower range, can cause measurable and highly significant endocrine disruption. Most of the studies rely on changes in gene expression (gene activation or suppression). The use of 'omics' will provide a deeper understanding of the effects of EDCs, and offer regulators a new approach to understanding the mechanisms of action. They are already an invaluable addition to a toxicologist's toolbox, and will be able to elucidate areas such as dose-response relationships. Omics will hopefully overcome some of the limitations inherent in traditional methods, although they are not without their own limitations. Their utility lies with where best to apply them to maximise their use.

2.1 What are omic technologies?

The terminology used to describe omic technologies is often confusing. People use the term 'genomics' in a very broad sense, and it often encompasses several scientific disciplines and technologies. The disciplines include genome sequencing; annotating genes to a particular function; studying gene expression at the transcript level (transcriptomics); studying protein expression at the protein level (proteomics); and the study of metabolite fingerprints (metabolomics). More detailed information can be found in a report entitled *Environmental genomics: an introduction* from the Environment Agency website (http://publications.environment-agency.gov.uk/pdf/SGENOMICS-e-p.pdf). The use of omics (including transcriptomics, proteomics and metabolomics) to study ecotoxicology is termed ecotoxicogenomics. Ecotoxicogenomics makes use of all the omic technologies and bioinformatics in the study of ecotoxicology, and is the term most will be familiar with in the context of endocrine disruption. It has been defined as the study of gene and protein expression in wild animal species that is important in responses to environmental toxicant exposures (Snape *et al*., 2004). Omics (hereafter referred to as genomics) generate large, complex datasets that require interpretation using sophisticated software. This type of analysis is called bioinformatics.

2.2 Transcriptomics

The transcriptome holds information on the qualitative and quantitative properties of mRNA (transcript), indicating the active components within a cell. The analysis of mRNA in a cell, tissue or organism is termed transcriptomics and it allows the monitoring of gene expression in a given physiological condition. Traditional methods in molecular biology analyse a small number of genes (1-10) in one experiment. Although informative, throughput is limited and a holistic picture of gene function is difficult to obtain. The use of DNA chips/microarrays enables the study of the expression of hundreds to thousands of genes (whole genomes) simultaneously, and is extremely powerful. A precursor of array technology was through major developments in large-scale sequencing, providing vast amounts of sequence information. Transcript levels of all predicted genes can now be measured simultaneously at several time points under specific conditions, to identify those genes that are either induced or repressed in relation to a control sample (Lander, 1999). The expression profiling of thousands of wild animal species in response to environmental toxicant exposure is termed ecotoxicogenomics (Snape *et al*., 2004).

Briefly, DNA sequences are immobilised onto a solid support such as a glass slide or nylon membrane. The mRNA is extracted from both the test and control sample, reverse transcribed to form cDNA, labelled and hybridised to the microarray. The microarray is scanned and a semi-quantitative measure of the extent of hybridisation between the control and test sample determined. Transcriptomics can either be used to determine the mechanistic action of a toxicant and identify genes and pathways affected by it, or it can be used in a predictive context. When used in a predictive context, the profiles of specific toxicants are compared to “signature” profiles of reference toxicants. Whatever context they are used in, these methods provide insights into the molecular mechanisms of chemicals and should enable us to identify effective biomarkers of exposure.

Despite these types of studies being crucial, it has been shown in yeast that mRNA and protein expression levels do not correlate (Gygi *et al*., 1999). Therefore, proteomic studies are also essential in order to understand cellular functions. For more information on DNA microarrays, see Jares (2006) where different DNA microarray applications developed by scientists in recent years are reviewed. In addition, Sobek *et al*. (2006) review developments in microarray technology and highlight crucial bottlenecks and future directions. Elsewhere, the use of microarray technology other than for investigating DNA and gene expression has gained importance. Applications include antibody, peptide, protein and cell arrays. Protein and antibody arrays are in the early phases of development, where the complexity of proteins creates problems for high-throughput production of the arrays (see Sobek *et al.* for a detailed review).

2.2.1 Real-time PCR

Publication guidelines require that microarray results be confirmed by independent gene expression methods and real-time PCR (RT-PCR), also known as quantitative (real-time) PCR (Q-PCR), is often the method of choice (see Klein, 2002, for applications and limitations of Q-PCR). It is used to validate/quantify the expression of differentially expressed genes. RT-PCR methods are based on the use of two chemistries. The first is the use of double-stranded DNA (dsDNA) binding dyes that intercollate with dsDNA. The second is the use of fluorogenic probes that fluoresce when hybridised to their target DNA. RT-PCR was improved by the introduction of probe-based chemistry, in that no signal is generated from mispriming or primer-dimer artefacts, reducing the incidence of false positives. Also, more than one reporter dye can be used which enables the detection of more than one target gene in a single reaction. When RT-PCR is combined with reverse transcription, it is used to rapidly quantify mRNA, facilitating the quantification of gene expression at a particular time in a particular tissue from low quantities of starting material. In the context of endocrine disruption, transcriptomics using a microarray approach would best be applied as a discovery tool to expose candidate toxic response genes. These genes could be used to develop cost-effective, sensitive RT-PCR diagnostic screening tools for endocrine disruption.

2.3 Proteomics

Proteomics is the study of the proteome. The proteome is the complement of proteins (encoded by the genome) expressed by an organism, tissue or cell, and it varies from one cell type to the next. Proteomics not only attempts to uncover a protein's specific function (each protein has a set function) but also how it interacts with other proteins. The technique also identifies the protein profile of each cell type and assesses protein

differences between healthy and diseased cells. Changes in the levels of expression of proteins are associated with stress, changes in metabolism, disease states, and so on.

There are two key steps to proteomics that provide the raw material for protein identification. The first is a high resolution protein separation method and the second is an accurate identification method. The separation method is dominated by two-dimensional polyacrylamide gel electrophoresis. Basically, the proteins from a cell or tissue are extracted and separated first in one direction (charge - isoelectric point) and then in a second (molecular weight). An alternative method for separating protein mixtures is to use a high resolution liquid chromatography technique, where separation is based on the difference in affinity of a protein towards a mobile phase compared to a stationary phase.

Once separated, targeted proteins are generally digested (chemically/enzymatically) and the resultant peptides analysed by mass spectrometry (matrix-assisted laser desorption ionisation time-of-flight (MALDi-TOF) mass spectrometry). A peptide mass fingerprint is produced and compared to a list of *in silico* generated peptides of known identity, to characterise the protein of interest. Further processing the sample can reduce uncertainties in identification. Each peptide fragment can be further fragmented into individual amino acid residues using tandem mass spectrometry (or MS/MS - the peptides are broken by collision with argon molecules). The amino acid information and peptide fingerprint can be combined to increase the accuracy of identification. For more detailed information on different separation and quantification techniques for protein analysis, see Klein (2002) and Hunzinger *et al.* (2006).

2.4 Metabolomics

Metabolomics is the newest of the genomic technologies, but because of its vast potential it is moving at a very fast pace. It is essentially the study of the metabolome, which represents the output that results from the cellular integration of the transcriptome and proteome (Joyce and Palsson, 2006). Metabolomics provides a list of metabolite (carbohydrate, lipid and amino acid) components in a cell, but is a functional readout of the physiological state of the cell at any point in time; in other words, it is the measure of an organism's phenotype. The goal of metabolomics is to characterise the biochemical differences between a biological control sample and a test sample (specific disease state or stress response). Changes in a disease state or stress response trigger changes at the molecular level, and subsequently the organism's phenotype becomes altered. Essentially it aims to identify, measure and interpret the complex time-related concentration, activity and flux of metabolites in biological samples such as cells, tissues, blood, urine, and saliva.

As with the other genomic technologies, data is generated using sophisticated techniques such as mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy and vibrational spectroscopic techniques. MS and NMR are the techniques most commonly applied since they can detect a wide range of metabolites (but not all) with relatively high specificity and reproducibility (Lin *et al.*, 2006). Each of the techniques used to analyse metabolites have limitations in quantification, scope and throughput with no one technique being effectively able to identify, measure and quantify the diverse range of metabolites and their fluctuations in cells with sufficient sensitivity. An integrated array of technologies is needed to address the entire spectrum of challenges for metabolomics.

Metabolomics has been reviewed by several authors including Rochfort (2005), who provides information on metabolomic methods and how the information they generate can be combined with other genomic datasets to improve our understanding of biological systems; Dunn *et al*. (2005) reviews the advantages and disadvantages of the different metabolomic techniques and how they have been applied and likewise Lin

et al. (2006) review the techniques and applications, however, their main focus is on applications in environmental toxicology.

3 Mechanisms of endocrine disruption

The main endocrine pathways under investigation involve oestrogen, androgen and thyroid hormones. EDCs mimic, block or modify the effects of natural hormones. Classically, EDCs have appeared to act via genomic mechanisms, where they bind to or prevent binding to the steroid receptors initiating a receptor-mediated effect. There is, however, evidence that some compounds do not act via a receptor-mediated mechanism. Receptor-independent pathways can involve alterations in the synthesis of hormones by modifications in the enzymatic pathway, or by direct changes in enzyme synthesis or in enzyme activity levels (Sanderson and van den Berg, 2003). The toxic effects of EDCs, however, are not confined to their hormonal actions. For instance, pesticides have been attributed to a mode of action as neurotoxins (Tilson and Kodavanti, 1997).

Many EDCs, when tested in vivo, fail to correlate with receptor binding and the observed physiological effect of endocrine disruption. For instance, nonylphenol and bisphenol A (BPA) are only weak oestrogen receptor agonists, but they have strong endocrine disrupting activity in vivo which suggests alternative mechanisms beyond the classical nuclear receptor binding mode. Indeed, recent studies have shown that BPA can act via non-genomic (non-nuclear) receptors to activate cell signalling pathways at very low concentrations. Picomolar to nanomolar concentrations of BPA can induce rapid intracellular Ca2+ changes (within 30 seconds), which lead to prolactin secretion (within one minute) in rat pituitary tumour cell subline GH3/B6/F10 (cells naturally express high levels of a membrane form of ER-α which are thought to mediate non-genomic actions) (Wozniak et al., 2005). The release of prolactin can initiate signalling cascades, leading to a variety of kinase activations (such as adenylyl cyclase production of cAMP leading to activation of protein kinase A, phospholipase C activation resulting in activation of protein kinase C, calmodulin activation of pathways leading to MAP kinase phosphorylation and more) that results in the phosphorylation status of cellular proteins changing. This can have subsequent rapid functional consequences (Wozniak et al., 2005).

The impact of EDCs can cause ED through a range of mechanisms by acting as:

- environmental oestrogens that lead to feminisation, such as methoxychlor, bisphenol A;
- environmental anti-oestrogens that neutralise sexual differentiation, such as dioxin, endosulfan;
- environmental androgens that cause masculinisation, such as tributyl tin (TBT);
- environmental anti-androgens that have feminising effects, such as vinclozolin, DDE;
- toxicants that reduce steroid hormone levels, such as fenarimol, endosulfan;
- toxicants that affect reproduction primarily through effects on the central nervous system, such as dithiocarbamate;
- others which, for example, alter thyroid hormone levels, aromatase activity, and so on.

A wide range of chemicals have been found or are suspected to be capable of disrupting the endocrine system, including:

- pesticides (such as DDT, vinclozolin, TBT, atrazine);
- persistent organochlorines and organohalogens (such as polychlorinated biphenols, dioxins, furans, brominated fire retardants);
- alkyl phenols (such as nonylphenol and octylphenol);
- heavy metals (such as cadmium, lead, mercury).

It has recently been reported that nitrates have the potential to be EDCs (Guillette and Edwards, 2005).

The range of mechanisms by which EDCs can act will mean that a number of test methods will have to be developed to adequately cover the different mechanisms that could be targeted by EDCs. Giesy et al. (2003) state that to accurately assess ecological risk, it is necessary to understand the mechanisms of action of a contaminant, and only with this understanding can effects of complex mixtures of contaminants be interpreted and appropriate thresholds developed to provide adequate protection.

3.1 Receptor-mediated genomic mechanisms of hormones

Hormone receptors form a class of ligand-activated proteins that, when bound to specific target sequences in genomic DNA, act as an on-off switch for transcription of nearby genes within the cell nucleus. These nuclear receptors are represented by a superfamily of steroid/thyroid/retinoid/orphan receptors that hormones bind to. Orphan receptors are classified as having no or as yet undiscovered ligands (Giguère, 1999). The receptors, the best known being the oestrogen receptor, play a critical role in the control of the development and differentiation of skin, bone, behavioural centres in the brain and the continual regulation of reproductive tissues. Thus, tissue-specific effects of hormones are mediated through variations in expression of the receptors. The oestrogen receptor alpha (ERα) in vertebrates, for example, after binding its ligand, will alter the transcription of target genes. This mechanism, which has received most of the attention in the area of endocrine disruption research, is via a “genomic” signalling pathway (“genomic” as it directly involves the steroid bound to the receptor acting directly on the cell’s DNA). It is a ligand-dependent mechanism of transcription that can be subdivided into classical and non-classical pathways.

3.1.1 Classical mechanisms: hormone response element-dependent

The classical mechanism of a steroid receptor action involves ligand binding to DNA and RNA in the cell’s nucleus to produce a protein and exert a biological function. For an effect to be seen at the level of the whole organism (where significant amounts of the appropriate protein must be produced), the process usually takes hours or even days (Lösel *et al.,* 2003). For a comprehensive review of the mechanisms of steroid hormone receptors, see Beato and Klug (2000). In a simple model, the receptor is inactive in the absence of a ligand in the cytoplasm of a cell. When a ligand diffuses into the cell, it displaces inhibitor proteins on the receptor (heat shock proteins) and binds to the receptor, specifically to the ligand binding domain (LBD). The receptor-ligand complex translocates to the nucleus where DNA binding takes place. The DNA

binding domain (DBD) of the receptor binds to specific hormone response elements (specific DNA sequence, such as oestrogen response elements (ERE)) in the promoter region of the target gene, and recruits a number of cofactors that facilitate transcription. The target genes are subsequently either up- or downregulated. As a result of the receptor-ligand interaction, a cascade of genes are expressed and coordinated subsequent to protein synthesis. This receptor-mediated mechanism of steroid action is central to the physiological characteristics observed in vertebrates resulting from exposure to EDCs.

3.1.2 Non-classical mechanisms (cross-talk): hormone response element-independent

Björnström and Sjöberg have found evidence of mechanisms of ER signalling that deviate from the classical model (2004; 2005). They review a number of studies which show that ERs can regulate transcription without binding to EREs, but instead are tethered through protein-protein interactions to a transcription factor complex that contacts the target gene promoter. This is the non-classical signalling pathway and the mechanism is referred to as cross-talk. It is a common mechanism for several nuclear receptors. Essentially, the transcription of genes are altered as a result of the ER altering the activity of other transcription factors such as AP-1, Sp1 or NF-κB, without binding to an ERE (Glidewell-Kenney *et al.*, 2005). Glidewell-Kenney *et al.* (2005) identified non-classical pathway genes that responded to 17β-oestradiol (E2), selective oestrogen receptor modulators, tamoxifen and raloxifene or the oestrogen antagonist ICI 182,780, after introducing a mutant form of a ERα (E207A/G208A) that does not bind to EREs, and introducing into ERα-negative breast carcinoma cells. Using microarray analysis, they identified 268 responsive non-classical ERα pathway targeted genes.

3.2 Receptor and non-receptor mediated non-genomic mechanisms of hormones

Genomic mechanisms of hormone action involve an interaction with an intracellular receptor together with a hormone response element or alternative transcription factor in the promoter region of the target gene. These mechanisms are typically slow and complex and as a result, can take hours to complete. In contrast, steroid hormones can mediate acute non-genomic mechanisms that cannot be accounted for by the activation of RNA and protein synthesis (Watson *et al*., 1995; Ruehlmann and Mann, 2000). The mechanisms of non-genomic action are not uniform and are insensitive to inhibitors of transcription and translation; a variety of modes have been described (Falkenstein *et al.*, 2000a and b). There are vast arrays of non-genomic actions (Simoncini and Genazzani, 2003) which include:

- G protein-coupled receptors (GPCRs), one of the best characterised mechanisms of non-genomic signalling;
- cell membrane ion channels, where one of the earliest observed rapid actions of steroids was the regulation of transmembrane ion fluxes;
- protein kinase signalling pathways, which include MAPK cascades, tyrosine and lipid kinases (P13K).

Through the use of such pathways, steroids can rapidly regulate multiple cellular functions and also modulate longer-term gene expression, protein and DNA synthesis (Simoncini and Genazzani, 2003).

Falkenstein *et al.* (2000a) propose the Mannheim classification scheme for non-genomic responses initiated by steroid hormones (for more detail, see Falkenstein *et al*., 2000a and b, and Lösel *et al*., 2003). They provide a review of the various actions of steroid hormones, focusing on rapid non-genomic effects. The authors discuss the different receptors mediating both genomic and non-genomic steroid action including progesterone, oestrogen, androgen, glucocorticoids and neuroactive steroid receptors. There are essentially six categories, with the main groups A and B differing in the requirement of a partner agonist to elicit a rapid response. Briefly, classification includes:

- AI – direct action with no receptor involvement, where non-genomic effects of steroids can be induced at high steroid concentrations by modulation of protein function, reflecting changes in membrane physicochemical properties.
- AIIa – direct action via classical intracellular (nuclear) receptors. Watson *et al.* (1995) have shown that oestradiol (E2) binds to a subpopulation of ERs located in the plasma membrane of GH3 pituitary tumour cells that release prolactin within one minute of treatment.
- AIIb – direct action via non-classical receptors. The majority of rapid effects of steroids on cellular signalling and function are thought to be mediated by membrane-associated receptors that are unrelated to the classical nuclear receptors (Zhu *et al*., 2003a and b).
- BI and BIIa – indirect action with no specific receptor involvement and indirect action via a classical nuclear receptor respectively. At the time of proposing the classification scheme, these two categories were hypothetical as there were no known examples.
- BIIb – indirect action where the steroid requires a partner agonist to mediate an effect via a non-classical receptor. Neuroactive steroids can rapidly alter the excitability of neurons by binding to membrane-bound receptors. The steroids act as a coagonist by augmenting the GABA-activated chloride currents.

In summary, three different mechanisms appear to exert effects via non-genomic actions (Lösel *et al*., 2003). The first is by classical steroid receptors, where the specificity of the ligand is the same for genomic and non-genomic actions; the second is via a non-specific effect at the membrane level requiring no receptor; and the third involves novel steroid receptors that are unrelated to the classical receptors.

Zhu *et al.* (2003a) claim to have cloned and characterised the first gene and corresponding protein unrelated to steroid nuclear receptors, where the gene has been shown to fulfil all the criteria for steroid receptor designation. They present convincing evidence of a non-genomic mechanism in which a steroid receptor located in a plasma membrane does not interact directly with DNA to initiate transcription. Instead, it is involved in a signal cascade event which initiates a more rapid response. Using a combination of protein purification, antibody screening and molecular approaches, the authors cloned a progestin membrane receptor (mPR) from *Cynoscion nebulosus*, a spotted sea trout. They demonstrated that the isolated receptor displayed all the criteria for designation as a steroid membrane receptor. These included:

- plausible structure, where computer modelling predicted the receptor to have seven transmembrane domains – a common feature of receptor structure;
- tissue specificity, where *in vivo* expression of the cloned protein was only detected in the brain and reproductive tissues (sites of progesterone production and secretion) using Northern blots;
- cellular distribution, where Western blot analysis confirmed its localisation in the plasma membrane fraction of oocytes and not in the cytosolic fraction;

- steroid binding, where using an *E.coli* expression system, the recombinant protein displayed saturable, displaceable specific progesterone binding compared to a control lacking the recombinant protein;
- signal transduction, where insertion of the cDNA coding region of the putative mPR into a mammalian expression vector and subsequent transfection into human breast cancer cells (MDA-MB-231) resulted in inhibition of cAMP production (with cAMP levels reduced within five minutes) and rapid and transient activation of the MAP kinase signalling pathway (which was dose dependent over a range of 10 to 1,000 nM) in response to progestin hormones in the breast cancer cells compared to control cells;
- hormonal regulation, where both the putative membrane receptor mRNA and protein were upregulated in ovarian tissues after incubation with 20β-S compared to control tissues incubated without hormones; and
- biological relevance, where Western blot analysis revealed that putative mPR levels varied according to the developmental stage of the oocyte, with highest levels during oocyte maturation. Similar results were obtained from field studies of fish captured on their spawning ground. To confirm this, antisense studies on the homologous mPR gene from zebra fish (sea trout oocytes are too fragile to work with), which has an amino acid sequence similarity of 80 per cent, were performed and the protein consistently blocked maturation of oocytes *in vitro,* providing more evidence of mPR involvement in progestin induction of oocyte maturation in teleosts (Zhu *et al*., 2003a).

In addition to demonstrating involvement in signal transduction, the cAMP experiments suggested that the receptor activates an inhibitory G protein which advocates it as a G protein-coupled receptor (GPCR). Inhibitory G proteins have been linked to signal transduction of rapid non-genomic actions in different cell types for testosterone and oestrogen (Lutz *et al*., 2000; Ho and Liao, 2002). In a subsequent study, Zhu *et al*. (2003b) identified and partially characterised 13 additional vertebrate genes from species including humans, mouse, pig, *Xenopus*, zebra fish and *Fugu* (Japanese pufferfish). Using phylogenetic analysis, α, β and γ forms of the receptor were identified.

3.3 Interaction between non-genomic and genomic actions

Despite the differences between genomic and non-genomic actions, there are instances where the two interact. Action of the thyroid hormone (TH) is mediated principally through the classical mechanism of action via nuclear receptors. Moeller *et al*. (2006) recently described an extranuclear mechanism of TH action in human fibroblasts, where TH-liganded thyroid receptor (TR)β interacts with a regulatory subunit of P13K (p85α) in the cytosol, causing activation of P13K and its downstream signalling cascade, sequential phosphorylation and activation of the serine/thyronine kinase Akt. Within minutes, the phosphorylated Akt is translocated into the nucleus. The process is rapid and independent of protein synthesis, typical of a non-genomic action, but its ultimate effect is genomic, with specific genes induced by this mechanism. This is an example of a hormone indirectly inducing a gene downstream of a non-genomic action. Oestradiol has been shown to activate the same signalling pathway in vascular endothelial cells, with subsequent upregulation of genes coding for enzymes or signalling molecules. The protein products of these genes directly affect cell functions and induce additional transcription (Pedram *et al*., 2002). Both of the studies demonstrating a link between non-genomic and genomics actions of hormones

used microarray analysis to determine target genes of the signalling cascade, with subsequent real-time PCR analysis to confirm microarray data.

4 Endocrine disruption in wildlife: molecular and genomic advances

The debate over ED has largely focused on gonadal steroids including oestrogens and androgens (although their role in amphibians is less well known). This is partly as a result of their link to infertility, breast cancer and low sperm counts in humans, but also because there are more and better-validated bioassays available to evaluate a response to the disruption of sex steroid function compared to the disruption of the thyroid system (Opitz *et al*., 2005). Most research, including genomics, has been conducted on fish. Recent interest in ED has extended beyond sex steroids and is now examining other hormones, including those associated with the thyroid, and pharmaceuticals. Reviews by Brucker-Davis (1998) and Rolland (2000) provide evidence from several studies, including field studies, confirming an association between environmental chemicals (pesticides and industrial chemicals) and thyroid disruption. Research in this area is increasing, especially in amphibians where the role of the thyroid is well understood.

A controversial issue surrounding ED is whether or not low-level exposure to EDCs can have adverse effects. Some scientists have found effects at low doses in laboratory experiments, while others have not been able to corroborate these findings. The controversy over atrazine and its effects in amphibians is a classic example (see Section 3.4.1). Some argue that traditional toxicological testing methods are not robust enough to pick up low-dose effects. This is an area where the application of genomics will see benefits, especially using gene expression analyses, because low-dose effects must involve changes in gene expression. This will be an important issue to resolve because of the presence of low levels of EDCs in the environment.

Wildlife populations are suspected to have been adversely affected by EDCs, where some of the potential impacts include (OECD, 2004 and references therein; Hayes *et al*., 2006):

- thyroid dysfunction in birds, amphibians and fish;
- decreased fertility in birds, amphibians, fish, invertebrates and mammals;
- decreased hatching success in birds, fish, alligators and turtles;
- gross birth defects in birds, amphibians, fish and turtles;
- metabolic abnormalities in birds, fish and mammals;
- behavioural abnormalities in birds;
- demasculinisation and masculinisation of fish, amphibians, alligators and birds;
- compromised immune system in birds, mammals and amphibians.

This list is by no means exhaustive. Using examples, this section will illustrate where genomic tools and techniques have been applied in order to unravel the molecular mechanisms of EDCs in fish and other wildlife species.

Investigations into the responses of individual genes to EDCs in fish and other wildlife species using genetic tools have begun, notably for biomarkers of oestrogen and androgen exposure (for example, mRNAs for vitellogenin (Bowman *et al.*, 2000) and spiggin (Kawasaki *et al.*, 2003)). However, studies on genes that control the central processes of growth, development and reproduction have been less forthcoming. Furthermore, a number of hormone-mimicking chemicals are known to have multiple

biological effects at various levels of biological organisation, which in turn means that they will have multiple pathways of action (and thus affect the expression of many genes (see Filby *et al.*, 2006)). Thus, there is a need for a more comprehensive approach than measuring transcription rates for single genes only, to fully understand the effect pathways of EDCs.

Whole genome sequencing projects have been initiated for a number of fish and other wildlife representatives, including the Japanese pufferfish (*Takifugu rubripes*), freshwater pufferfish (*Tetraodon nigroviridis*), Japanese medaka (*Oryzias latipes*), zebra fish (*Danio rerio*), little skate (*Leucoraja erinacea*), soil nematode (*Caenorhabditis elegans)*, domestic chicken (*Gallus gallus*), common fruitfly (*Drosophila melanogaster*), African clawed frog (*Xenopus laevis*), purple-spined sea urchin (*Arbacia punctulata*), water flea (*Daphnia pulex*) plus many others. In addition, large expressed sequence tag (EST) sequencing projects are ongoing for numerous fish and other species, including the three-spined stickleback (*Gasterosteus aculeatus*), channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis niloticus*), Atlantic salmon (*Salmo salar*), fathead minnow (*Pimephales promelas*), roach (*Rutilus rutilus*), water flea (*Daphnia magna*), ambystomatid salamander (*Ambystoma mexicanum*) and marine opisthobranch mollusc (*Aplysia californica*) plus more. Genome sequencing efforts have largely focused on the so-called 'model organisms'. However, many laboratories throughout the world are working with non-model species that offer a role as sentinels for ecotoxicology research. Functional genomic investigations of new and potentially more relevant species can be quickly implemented through EST sequencing projects. In addition, one species cannot satisfy all experimental requirements, thus the need for alternatives.

Genome sequences and EST databases for example on representative fish species come from different environments and ecological niches. They cover both scientific and commercial interests, as some fish are widely used as vertebrate models, such as zebra fish (model for studies of vertebrate development, developmental biology, and some human genetic diseases), and stickleback (model to study adaptive evolution) or are commercially valuable, such as channel catfish, rainbow trout, tilapia and Atlantic salmon. In addition, most of these fish species have been widely used in ecotoxicology studies to assess the effects of EDCs on health, reproduction, behaviour, and so on. The genetic information currently available for fish in particular, but also for other wildlife, is expanding. This, together with the recent advances in molecular techniques, now makes it possible to study the effects of EDCs on the expression of many genes simultaneously, allowing for molecular pathways (mechanisms) to be more readily identified. Additionally, information on gene expression could potentially provide valuable endpoints in the testing and monitoring of EDCs.

4.1 Fish

Exposure to EDCs in both freshwater and marine environments has been shown to alter the reproductive physiology and morphology of fish, resulting in female-specific proteins induced in male fish, skewed sex ratios, reduced sperm counts and an increased prevalence of intersexuality in males, or induction of gonopodia in females.

Extensive studies on wild populations of roach, *Rutilus rutilus* (a cyprinid fish related to the carp) in UK rivers demonstrated that EDCs in sewage treatment work (STW) effluents are capable of disrupting the sexual development of these fish, by inducing the transformation of testes into ovary-like structures containing oocytes and/or female-like reproductive ducts (Jobling *et al.*, 1998; Nolan *et al.*, 2001). The incidence of

disruption was positively correlated with the amount of STW effluent discharged into the waters inhabited by those fish (Jobling *et al.*, 1998).

Intersex in wild fish has now been reported in a number of fish species and in diverse geographical locations including Germany (bream, *Abramis brama*) (Hecker *et al.*, 2001), France (Minier *et al.*, 2000), Italy (barbel, *Barbus plebejus*) (Vigano *et al.*, 2001), Denmark (roach) (Christiansen *et al.*, 2000), and United Kingdom (gudgeon, *Gobio gobio*) (van Aerle *et al.*, 2001). The observed abnormalities were related to exposure to STW effluents. In the US and other developed countries, androgenisation of fish populations living downstream of industrial and municipal effluent discharge has been reported in a number of species, including the mosquito fish (*Gambusia holbrooki*) (Parks *et al.*, 2001); viviparous eelpout (*Zoarces viviparous*) (Larsson *et al.*, 2002); and fathead minnow (*Pimephales promelas*) (Orlando *et al.*, 2004). The effects observed include alteration of secondary sex characteristics in female mosquito fish (Parks *et al.*, 2001), alteration in gonad development in both male and female fathead minnow (Orlando *et al.*, 2004) and alterations of the sex ratios of populations of eelpouts (Larsson *et al.*, 2002). A variety of other chemicals have been found in STW effluents, including polycyclic aromatic hydrocarbons (PAHs; see Johnson *et al.*, 1998) and organochlorine pesticides (see Tyler *et al.*, 1998 and references therein), and these have been shown to be associated with a variety of physiological disruptions in wild fish populations.

Together, these field observations have resulted in a directed research effort into the effects of suspected chemicals on fish under controlled laboratory conditions. A large number of laboratory studies, and to a lesser extent studies on wild populations, have tried to assess the impact of EDCs on reproduction and development in fish and other wildlife species, and the likely impacts on wild populations. Until recently, less attention has been directed at identifying the mechanisms of disruption. Understanding these mechanisms is fundamental to establishing how chemicals cause the disruptions and indeed, to assigning causality of the phenotypic alterations observed to culprit chemicals. As previously mentioned, almost all pollutants that adversely affect humans and wildlife will do so by interfering with gene expression.

4.1.1 Oestrogenic chemicals

Many EDCs are oestrogenic in nature: these are the major contaminants in effluents from sewage treatment works (STWs) that discharge into rivers in the UK and more widely in Europe, such as in the Netherlands (Vethaak *et al.*, 2005) and Norway (Knudsen *et al.*, 1997), and indeed globally, such as in the United States (Folmar *et al.*, 1996) and China (Ma *et al.*, 2005).

Among these compounds are natural and synthetic steroid oestrogens, phyto- (plant) and myco- (fungi) oestrogens, and a wide variety of man-made industrial compounds, such as plasticisers, surfactants, bisphenol A (BPA), and various pesticides and herbicides (reviewed in Tyler *et al.*, 1998).

Exposure to STW effluents has been shown to result in an increased concentration of vitellogenin (Vtg; a biomarker of oestrogen exposure) in male fish, increased concentrations of 17β-oestradiol (E2) in the plasma of males and increased concentrations of testosterone in the plasma of females (Jobling *et al.*, 1998, 2002a). Moreover, sperm production, release, motility and the ability of sperm to successfully fertilise eggs and produce viable offspring were all found to be reduced in intersex roach from UK rivers compared with normal male fish, and they were negatively correlated with their degree of feminisation (Jobling *et al.*, 2002b). Together, these

observations raised concerns that environmental pollutants have the potential to affect the sustainability of wild fish populations. The process of gonadal sex differentiation and development is controlled by steroidal oestrogens and androgens (Conover *et al.*, 1981; Patino *et al.*, 1996; Strussmann *et al.*, 1996) and it is well established that exposure of male fish embryos to high concentrations of steroidal oestrogens can induce gonadal feminisation, even in gonochoristic (single sex) species (Yamamoto, 1953; Pandian *et al.*, 1995). In the medaka (*Oryzias latipes*) (Shibata *et al.*, 1988) and carp (*Cyprinus carpio*) (Gimeno *et al.*, 1998), germ cells maintain a bipotential even after gonadal differentiation, and exposure to high concentrations of oestrogens can result in feminisation in sexually mature adult males. The plasticity of gonadal development in fish, which contrasts with the more stable patterns found in higher vertebrates, may make fish potentially more susceptible to EDCs that interact with the sex steroid hormone pathways. Laboratory studies have shown that exposure of carp and medaka during early life-stages to chemicals that mimic oestrogens, including the alkylphenols, octylphenol (Gray *et al.*, 1999), p-nonylphenol (Gray *et al.*, 1997), and the pesticides, beta-hexachlorocyclohexane (Wester *et al.*, 1986) and methoxychlor (Nimrod *et al.*, 1998) can also disrupt gonad development, including the duct development and/or the presence of oocytes in the testes of males. In most of these studies employing steroidal oestrogens and their mimics, the effects seen only occurred at concentrations far in excess of those found in the aquatic environment. More recently, however, laboratory studies have shown that when fish are exposed during specific windows (periods) of early development, for example during the period of sexual differentiation, much lower concentrations (including concentrations found in some aquatic environments) can induce oocytes in the testes and/or disrupt the duct (Metcalfe *et al.*, 2001; van Aerle *et al.*, 2002).

Effects of oestrogenic EDCs on the expression of genes involved in reproductive development and function in fish have been investigated, including on the pituitary hormones, FSH and LH (Harris *et al.*, 2001), the enzymes P450 aromatase A and B that mediate the conversion of testosterone into oestrogen (Fenske *et al.*, 2001; Halm *et al.*, 2001), oestrogen receptors (Sabo-Attwood *et al.*, 2004; Filby *et al.*, 2005), and genes controlling sexual differentiation (Schulz *et al.*, 2006). These studies have focused on single genes, and have shown that these genes can be used very effectively to signal for subsequent feminised phenotypes.

A number of recent studies using quantitative real-time PCR and gene array technologies have started to make use of genomic resources to investigate oestrogenic effects in fish, with promising results.

In a study employing real-time PCR by Filby *et al.* (2006), the effect of E2 was studied on the expression of a suite of genes which interact to mediate growth, development and thyroid and interrenal function (growth hormone (GH), GH receptor (GHR), insulin-like growth factor (IGF-I), IGF-I receptor (IGF-IR), thyroid hormone receptors α (TRα) and β (TRβ) and glucocorticoid receptor (GR)) together with the expression analyses of sex steroid receptors and ten other genes centrally involved in sexual development and reproduction in fathead minnow (*Pimephales promelas*). Exposure of adult fhm to 35 ng E2 per litre for 14 days induced classic oestrogen biomarker responses (hepatic oestrogen receptor one and plasma Vtg), and affected the reproductive axis, feminising 'male' steroidogenic enzyme expression profiles and suppressing genes involved in testes differentiation (Filby *et al.*, 2006). However, E2 also triggered a cascade of responses for genes that mediate growth, development and thyroid and interrenal function (GH, GHR, IGF-I, TRα and $\beta$and GR), with potential consequences for the functioning of many physiological processes, not just reproduction. The molecular responses to E2 were complex, with most genes showing differential responses between tissues and sexes. For example, IGF-I expression increased in the brain but

decreased in the gill following exposure to E2, and responded in an opposite way in males compared with females in liver, gonad and pituitary (Filby *et al.*, 2006). These findings demonstrate the importance of developing an integrative understanding of endocrine interactions to unravel the mechanisms of environmental oestrogen action and predict the health consequences likely to arise from exposure to these chemicals.

Larkin *et al.* (2003) used a low density gene array containing 30 oestrogen-responsive genes to demonstrate that EDCs which mimic oestrogens exhibit unique genetic fingerprints. In this study, sheepshead minnow (*Cyprinodon variegatus*) were exposed to ethinyloestradiol (EE2) (109 ng/L) and diethylstilbestrol (DES; 100 ng/L) for four days, to E2 (65.14 ng/L) and *p*-nonylphenol (11.81 ug/L) for five days, and to methoxychlor (5.59 ug/L) and endosulfan (590.3 ng/L) for 13 days. Liver samples were analysed for alterations in gene expression and in total six genes, including *vtg1*, *vtg2*, and *esr1* (oestrogen receptor α), were similarly upregulated after exposure to all oestrogenic chemicals (apart from endosulfan). In total, three genes were down-regulated (transferrin, beta actin and AMBP) (Larkin *et al.*, 2003a). Other studies employing low density gene arrays have also shown that exposure of fish to oestrogenic chemicals (and at environmentally relevant concentrations) alters the expression of a cascade of genes known to be involved in reproduction, as well as genes encoding for novel and/or unknown proteins (for example, largemouth bass (Larkin *et al.*, 2002, 2003b) and plaice (Brown *et al.*, 2004)).

Scaling up further and using a microarray (containing 16k oligonucleotides), Santos *et al*. (2006) investigated genome level responses and the effects of EE2 on the gonads of breeding populations of zebra fish. In those studies, breeding adult zebra fish exposed to environmental concentrations of EE2 (5 ng/L) reported a decrease in the fecundity of the populations, a decrease in sperm motility and an increase in plasma Vtg in males (Santos *et al.*, 2007a). Microarray analyses of the gonads of these fish revealed that this concentration of EE2 induced significant changes in the expression of many genes in ovaries and testes. Gene ontology analysis of the affected gene lists revealed that in the gonads, the biological processes *cell cycle*, *mitochondrion organisation and biogenesis*, and *energy pathways* were particularly affected by the treatment in females whereas in males, *protein modification*, *protein metabolism* and *growth* were the biological processes most affected by the treatment. Alterations in these biological processes may be responsible, at least in part, for alterations in the number of eggs produced by females exposed to 5 ng/L EE2, decrease in sperm quality in males and induction of the synthesis of oestrogen-dependent proteins (Santos *et al.*, 2007a).

4.1.2 Androgenic chemicals

Several studies have shown that androgens or molecules with androgen-like activities are present in STW effluent, with concentrations ranging from 2 to 4,033 ng/L of dihydrotestosterone (DHT)-equivalents (Kirk *et al.*, 2002; Thomas *et al.*, 2002). Furthermore, in river water, concentrations of androgens have been reported up to more than 100 ng/L (androstendione; Jenkins *et al.*, 2001; Durhan *et al.*, 2002). Wild fish living downstream of some STWs effluents have been shown to have alterations in their reproductive function, consistent with androgenic exposure (Parks *et al.*, 2001; Larsson *et al.*, 2002; Orlando *et al.*, 2004). Investigations into the molecular alterations caused by androgenic exposure in the laboratory are ongoing and are trying to unravel the mechanistic pathways leading to the phenotypic alterations observed. Exposure to 17β-trenbolone (TRB; a synthetic steroid that is extensively used in the United States as a growth promoter in beef cattle) in the fathead minnow resulted in reduced fecundity following exposure to concentrations greater than or equal to 0.027 μg/L and alterations in the plasma concentrations of sex steroids and vitellogenin in females

(Ankley *et al.*, 2003). TRB was clearly androgenic *in vivo* at these concentrations, as evidenced by the *de novo* production in females of dorsal (nuptial) tubercles, structures normally present only on the heads of mature males. In males, TRB caused alterations in the reproductive physiology only at concentrations much higher than those producing effects in females and these included decreased plasma concentrations of 11-ketotestosterone (11-KT) and increased concentrations of E2 and vitellogenin (41 μg/L) (Ankley *et al.*, 2003).

Exposure of juvenile and sexually mature adult fathead minnows to 17α-methyltestosterone (MT) has been shown to produce both androgenic and oestrogenic effects (Ankley *et al.*, 2001; Zerulla *et al.*, 2002). The suite of effects observed in males included ovipositor development, vitellogenin production, and decreased plasma testosterone and 11-KT concentrations. Females displayed relatively rapid onset of nuptial tubercle formation, cessation of spawning, and decreased plasma concentrations of testosterone and E2. The binding affinity of MT for the oestrogen receptor is very low and could not explain the oestrogenic effects observed. In a separate study, exposure of fathead minnow to MT using a gonadal recrudescence assay resulted in an induction of both androgenic and oestrogenic effects, with females generally more affected by MT than males. Atretic follicles and male-specific sex characteristics (androgenic effect) were induced in females at or above 0.1 and 1 ug/L MT, respectively (Pawlowski *et al.*, 2004). An inhibitory effect on ovary growth occurred at an exposure concentration of 50 ug/L MT.

In the fish breeding studies, concentration-dependent reductions in egg number and fertilisation rate and increases in abnormal sexual behaviour in females were observed at exposure concentrations of 5 ug/L MT or above (Pawlowski *et al.*, 2004). Similar results were obtained after exposure of zebra fish to different concentrations of MT (Andersen *et al.*, 2006). At a concentration of 4.5 ng MT/L, a significant increase of plasma Vtg was observed (compared to solvent control group), whereas exposure to higher concentrations of MT (ranging from 6.6 to 62.3 ng/L) did not result in an increase in Vtg levels. Furthermore, endogenous 11-KT and testosterone levels decreased significantly in a concentration-dependent manner in response to MT exposure, and the lowest effective concentrations were 6.4 and 8.5 ng MT/L, respectively (Andersen *et al.*, 2006). In follow-up studies, an explanation for these paradoxical results (oestrogenic response after androgen exposure) was found, where it was shown that MT can be aromatised in fish and converted into 17α-methyloestradiol, a potent oestrogen (Hornung *et al.*, 2004). Together, these findings show that androgenic chemicals appear to be a major environmental issue in some parts of the world, notably for specific effluent types (such as paper and pulp mill effluents and concentrated animal feeding operation (CAFO) waste waters).

In order to establish the molecular mechanisms of disruption and understand the physiological pathways affected by these chemicals, the alterations of gene expression profiles following exposure need to be documented. Blum *et al*. (2004) investigated the effect of two natural androgens, DHT and 11-KT, on adult largemouth bass using a cDNA array containing 296 genes (132 oestrogen-responsive genes and 164 androgen-responsive genes) (Blum *et al.*, 2004). Adult male largemouth bass were treated with 62.5 ug/g DHT or 2 ug/g 11-KT or vehicle (DMSO) (n = five individuals per treatment) and liver samples were analysed for changes in gene expression. Several genes were induced by both treatments including *vtg2*, spermine-spermidine-N1-acetyltransferase (SSAT), warm water acclimatisation protein, rhamnose binding lectin, and two ESTs named seasonal (SNL) 64, and P4_H07. A number of genes were downregulated by both androgens including the LDL receptor, retinoic acid receptor interacting protein, VTG receptor, and two ESTs called SNL51 and GP3-11C. While the patterns of regulation appeared similar for both androgens, some specific differences

were observed. For example, only DHT increased ATPase 6, Tata box binding protein and ESTs SNL56, SNL62, P4_C04, and 97-8 and decreased apolipoprotein E, glutathione peroxidase III, haptoglobin, IGF-I, and hepcidin. Conversely, 11-KT stimulated the expression of solute carrier protein family member 25a5, pituitary tumour transforming protein, and ESTs DHT64, SNLF15, P4_E12, and downregulated cystatin, ribosomal protein Sa, and ESTs SNL55, SNL11, P4_E09, and P4_F11 (Blum *et al.*, 2004). The differences between the effects of these two androgens on gene transcription may reflect their endogenous activity (since both have specific functions in fish) or may be due to differences in binding affinities of the two fish androgen receptors, but this is not clear from the current androgen receptor literature (Blum *et al.*, 2004).

4.1.3 Thyroid hormone disrupting chemicals

Thyroid hormones are important in growth, development and the maintenance of appropriate cellular metabolism in vertebrates. Specific mechanisms of action of EDCs that act on the thyroid axis include alterations of thyroid hormone (TH) synthesis, TH transport, TH elimination, neuro-endocrine (H-P) axis regulation and TR expression and/or function (OECD, 2004). Thyroid hormone (T_3) affects a range of biological processes including differentiation, growth and metabolism. For a comprehensive review, see Yen (2001) and references therein for a description of both genomic and non-genomic mechanisms of thyroid action and subsequent mediated molecular mechanisms of action of TRs. Essentially, since the cloning of the first TR around 20 years ago, there has been an explosion in research on the molecular mechanisms of TR action. Like steroid hormones, two major isoforms, TRα and β, exist in fish, amphibians, chicken, mice, rats and humans (Yaoita *et al*., 1990; Marchand *et al*., 2001). Both isoforms bind T_3 and mediate TH-regulated gene expression. Regulation of TR mRNA is isoform and cell type dependent. In contrast to steroid hormone receptors that bind as homodimers to palindromic HRE, TRs bind TREs as monomers, homodimers and heterodimers, though the role of monomer and homodimerisation is not as well understood. Studies of heterodimerisation show that TRs heterodimerise with RXRs and bind their cognitive ligand *9-cis*-retinoic acid with high affinity.

Various EDCs have been shown to interfere with thyroid function in fish, including perchlorate, which has been shown to inhibit the uptake of iodide into the thyroid gland (resulting in a decrease in iodine-based thyroid hormone synthesis). Perchlorate contamination is widespread in the United States and concentrations have been measured up to 3,700 and 480 mg/L in ground and surface waters respectively, in several US states (Liu *et al.*, 2006). Exposure of zebra fish to 10 mg/L sodium perchlorate for 30 days resulted in major alterations in the structure of the thyroid gland (Liu *et al.*, 2006). Studies investigating the effects of this chemical on reproductive function in the mosquito fish found that similar concentrations to those found to substantially affect thyroid function in this species, did not have significant deleterious effects on reproduction (Park *et al.*, 2006). This indicates that this chemical specifically acts on thyroid function and highlights the need for integrative approaches to understand the specific effects of exposure to environmental chemicals on the biology of the whole organism.

Various studies investigated the effects of EDCs on genes regulating thyroid function, but the effects observed were not linked to specific thyroid disruption mechanisms and no functional link was established between gene expression alterations of those genes and corresponding phenotypic endpoints (for example, Filby *et al.*, 2006). There is a need for more research in this area, especially where alterations on thyroid gland morphology are linked to alterations in gene expression.

4.1.4 Organochlorine pesticides

The ability of organochloride pesticides (OCPs) to induce alterations in the endocrine homeostasis has been demonstrated in many studies in the last five decades (Tyler *et al.*, 1998). Its continued use as pest control in agriculture and also as disease control in tropical countries is unavoidable, given the needs of food production to support an ever-growing human population and the need to protect against diseases such as malaria. It is now known that many of these chemicals are persistent in the environment and are able to bioaccumulate and bioconcentrate in the food chain, and these factors increase the risk they pose to environmental and human health (Tyler *et al.*, 1998). Examples of OCPs that have been shown to disrupt the endocrine function in fish include pentachlorophenol (Dorsey *et al.*, 2004), endosulfan (Dutta *et al.*, 2006), and methoxychlor (Thorpe *et al.*, 2001).

Garcia-Reyero and colleagues (2006) studied the effects of long-term exposure of largemouth bass to the organochlorine pesticides, *p,p'*-DDE and dieldrin, on gene expression using real-time PCR. In this study, the expression of a suite of genes involved in reproduction was measured and these genes could be divided into three categories: receptor-related genes (*esr1, esr2a and esr2b*, androgen receptor (*ar*), vitellogenin (*vtg*)); genes involved in hormone synthesis (steroidogenic acute regulatory protein (*star*) and *cyp19*); and genes involved in hormone metabolism (*cyp3a68, cyp3a69*). *p,p'*-DDE altered the expression of genes involved in all three categories, and the resulting gene expression patterns suggested that it acted as a weak oestrogen. *p,p'*-DDE also inhibited the expression of the androgen receptor, and altered genes involved in the synthesis of endogenous hormones as well as their metabolism. Dieldrin acted mainly to downregulate the expression of mRNA for some of the hormone receptors, though it appeared not to act as an oestrogen. Dieldrin also altered mRNAs for proteins involved in hormone synthesis and metabolism. However, it was not clear which changes were caused by the chemicals and which were compensatory responses driven by regulatory input from the reproductive axis (Garcia-Reyero *et al.*, 2006).

4.1.5 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that have widely been shown to affect reproduction in aquatic organisms (Hall *et al.*, 1991; Johnson *et al.*, 1998). PAHs have been shown to cause deleterious effects on ovarian growth and spawning success. In addition, one of the most consistently reported reproductive effects of exposure to PAHs (both in the laboratory and in the field) is a decrease in circulating levels of E2 (see, for example, Thomas, 1988). For example, exposure of Atlantic croaker during ovarian recrudescence to a model PAH, benzo[*a*]pyrene (B[*a*]P), via i.p. injection resulted in decreased ovarian growth as well as lower circulating levels of testosterone and E2 (Thomas, 1988). Several mechanisms have been proposed to explain the effect that PAHs have on E2 levels including: (1) blocking of the E2-oestrogen receptor complex from binding to DNA by the arylhydrocarbon receptor (AhR)/AhR nuclear translocator protein (ARNT) complex; (2) inhibition of P450 enzymes; (3) increased catabolism of E2 by P450 enzymes; (4) alteration of transcription of steroidogenic enzymes critical to the production of E2 (see Hoffmann *et al.*, 2006 and references therein).

Hoffman and Oris investigated the effect of exposure of immature zebra fish to B[*a*]P (at 1.3 and 3.0 ug/L for 56 days) on a suite of genes involved in reproduction, including gonadotropins (*follicle stimulating hormone* (*fsh*) and *luteinising hormone* (*LH*)),

steroidogenic enzymes (*cyp11a1, cyp17, cyp19a1, cyp19a2 and hsd20b*), oestrogen receptor (*esr2*) and vitellogenin (*vtg*), which were measured using real-time PCR (Hoffmann *et al.*, 2006). This suite of genes is involved in regulating the development of the gonads, formation of the gametes, and the synthesis of endogenous steroids (oestrogens and androgens). In addition, cytochrome P450 1A1 (*cyp1a1*; involved in detoxification of chemicals) was also measured in the liver and heads as an indicator of exposure to B[*a*]P. A reduction in total egg output was observed in B[*a*]P-exposed fish, as well as a decrease in gonadosomatic index (GSI) in fish exposed to 3.0 ug/L B[*a*]P. A significant increase in *cyp1a1* expression in the heads as compared to the control was observed, whereas no significant difference in *cyp1a1* was detected in livers. A significant increase in *hsd20b* mRNA occurred in heads and pre-vitellogenic oocytes from fish exposed to 1.5 and 3.0 ug/L as compared to the controls. *cyp19a2* and *vtg* were significantly increased following exposure to 3.0 ug/L in the heads and liver, respectively. No effects on the expression of *fshb*, *lhb*, *cyp19a1* or *esr2* were observed (Hoffmann *et al.*, 2006).

4.1.6 Mixtures of chemicals

The main aim of developing research programmes in environmental genomics is to understand the biological consequences of exposure of wild organisms to environmental mixtures of chemicals (such as effluents). Despite this, very little is known on the alterations of gene expression profiles caused by exposure to such mixtures, partly because of the complexity of performing such studies and interpreting the results. In the USA, Denslow *et al*. initiated work of this nature and exposed largemouth bass to a range of paper mill effluent concentrations (10 to 80 per cent) in large flow-through tanks for varied periods of time including seven, 28 or 56 days (Denslow *et al.*, 2004). Plasma hormone levels in males and females and plasma Vtg in females decreased with dose and time. Gene expression patterns (determined by differential display reverse transcription PCR) for male fish remained largely unchanged following exposure, except for the induction of a small suite of genes, including *cyp1a*, which encodes for a protein involved in chemical detoxification. In contrast, exposure of females to these effluents resulted in upregulation of *cyp1a* that was accompanied by a generalised downregulation of genes normally expressed during the reproductive season. These anti-oestrogenic changes were in agreement with previous studies of bass exposed to these effluents, and may result in decreased reproductive success in affected populations (Denslow *et al.*, 2004).

In the UK, European flounders captured in two estuaries with distinct contaminant profiles were analysed for impacts of PAHs, heavy metals and oestrogenic compounds using a battery of biomarkers such as hepatic ethoxyresorufin-*O*-deethylase (EROD) activity (a highly sensitive indicator of contaminant uptake in fish), metallothione (a biomarker for heavy metal exposure) and plasma VTG, along with measurements of gene expression (real-time PCR assays for *cyp1a*, metallothionein and vitellogenin). This study established that gene expression measurements using real-time PCR were a valid and cost-effective means of assessing the impact and type of exposure in this species (George *et al.*, 2004).

Ongoing work in the UK is analysing the alterations in gene expression profiles of suites of genes controlling endocrine function, following exposure to STW effluents in the fathead minnow, using real-time PCR. This approach is likely to result in a better understanding of the effects of exposure on endocrine function, along with identifying a suite of novel gene markers indicative of specific pathways affected by exposure. In parallel, similar studies in roach are employing genome-wide array technologies as well as detailed real-time PCR analysis of a small suite of gene targets, to assess the effects of life-long exposure to single environmental chemicals and STW effluent. This

work is likely to result in a better understanding of the physiological alterations caused by exposure and the mechanisms of disruption, along with generating suites of biomarkers for environmental monitoring (Filby *et al*., 2006c; Lange *et al*., 2006; both in preparation).

4.1.7 Gene expression signatures

Gene expression signatures could be developed for a limited range of aquatic or terrestrial organisms and such information used to identify the mechanism(s) of action of a single chemical or complex environmental mixtures (Snape *et al.*, 2004). Recently, a few studies were published in which fish were exposed to a variety of model toxicants, including EDCs and oxidative stressors, while an attempt was made to identify unique gene expression patterns (Hook *et al.*, 2006; Moens *et al.*, 2006). Hook *et al*. exposed rainbow trout to a variety of model toxicants, including EE2 (a potent synthetic oestrogen), TRB (a potent synthetic androgen), 2,2,4,4'-tetrabromodiphenyl ether (BDE-47; a flame retardant suspected of having thyroid disrupting properties), B[*a*]P (a carcinogen and genotoxicant), diquat (an aquatic herbicide and potent oxidative stressor), and chromium VI (a metal and oxidative stressor). The fish were exposed to EE2 (50 ng/L), TRB (1 ug/L), and B[*a*]P (1 ug/L) for seven days using a flow-through exposure system. BDE-47 was given orally (approx. 500 ug/kg) and diquat and chromium VI were injected intra-peritoneally (500 ug/kg and 25 ug/kg, respectively). Liver samples were collected and analysed using a 16k cDNA microarray and the results showed that the overall patterns of gene expression were unique to each model toxicant. Furthermore, the functions of genes with altered expression also had a unique pattern for each of the contaminants tested. While each toxicant signal was unique, the degree of overlap correlated with function. For example, the endocrine active compounds (EE2 and TRB) had more similar expression patterns to each other than to the other contaminant classes. Hierarchical clustering of gene expression data showed that unique expression profiles were generated for each compound, that these profiles were non-random, and that compounds with similar function were more tightly grouped together than compounds with disparate function (Hook *et al.*, 2006).

In another study, carp (*Cyprinus carpio*) were exposed to 14 OECD-recommended reference EDCs (E2, EE2, 4-nonylphenol, bisphenol A, tamoxifen, MT, 11-KT, dibutyl phthalate, flutamide, vinclozolin, hydrocortisone, CuCl2, propylthiouracil, and a mixture of L-triiodothyronine and L-41 thyroxine) with various mechanisms of action. The subsequent gene responses were measured using a microarray containing 960 cDNAs (Moens *et al.*, 2006). Each compound produced its own unique expression pattern on the array and a small gene set, consisting of 12 informative genes, was identified to discriminate between the different compounds. Although some compounds preferentially clustered with compounds outside their presumed mode of action, some marked expression similarities were observed between groups of analogous compounds (Moens *et al.*, 2006).

Overall, these studies suggest that the use of genomic tools and techniques have great promise to screen new and existing chemicals on their endocrine disruptive potential, and to identify distinct classes of endocrine disruptive compounds.

4.1.8 Multi-species arrays

Recently, a few studies have shown that gene arrays can be employed for the analysis of gene responses to EDCs in multiple fish species. For example, von Schalburg *et al*.

(2005) have shown the potential of a 16k cDNA salmonid microarray for a wide range of teleosts, including four salmonid species (Atlantic salmon, *Salmo salar*; rainbow trout, *Oncorhynchus mykiss*; chinook salmon, *Oncorhynchus tshawytscha*; and lake whitefish, *Coregonus clupeaformis*) and one member of the order Osmeriformes (rainbow smelt, *Osmerus mordax*). In this study, the average percentage binding of liver targets to the microarray chip ranged from 30 to 63 per cent, and there were no significant differences in the percentage of targets that bound to the 16K microarray for the four salmonids examined. However, rainbow trout targets did consistently show higher overall binding to the microarray; the reasons for this efficiency were not clear (von Schalburg *et al.*, 2005).

More recently, a multi-species gene array containing 95 genes known/suspected to play key roles in sexual development and function in vertebrates was constructed with cDNA derived from three fish species (*Danio rerio, Pimephales promelas, Rutilus rutulis*) to investigate the mechanistic pathways of sexual disruption (van Aerle *et al.*, 2006). Sexually mature, pair-breeding fathead minnow (*Pimephales promelas*) were exposed under flow-through conditions for 21 days to 0, 51, 94, 178, and 497 ug/L fenarimol using the fathead minnow adult pair-breeding assay (see Thorpe, 2006, for further details). Gene expression analysis in the gonads (ANOVA) identified eight genes that were differentially expressed in females and seven that were differentially expressed in males after exposure to fenarimol ($p < 0.05$). When multiple probes (derived from different fish species) were available for a single gene, the expression profile measured using each of the probes correlated well (as determined by cluster analysis, for example, *bactin1*, *ef1a*, *cyp19a1a*) (van Aerle *et al.*, 2006).

Thus, initial investigations into the use of gene arrays to study the effects of EDCs on gene expression in multiple fish species are looking promising. Multi-species arrays appear particularly useful for measuring gene responses in fish for which there is limited gene information.

4.2 Aquatic mammals

Mammals are represented by over 4,500 species (Vaughan *et al.* 2000). They have a vast diversity of forms and exploit a large variety of ecological niches and life history strategies. The majority have young that develop inside the uterus attached to a placenta. Feeding strategies are thought to directly affect exposure to EDCs (Fossi and Marsili, 2003). Suckling of young is thought to represent an important exposure route to chemical contaminants during critical periods of development. As mammals are top predators, persistent organochlorines such as PCBs and DDT that tend to accumulate down the food chain are passed down through generations.

Endocrine disrupting effects in mammals have mainly been reported in aquatic environments and both reproductive and non-reproductive effects have been associated with organochlorines. Reproductive effects include masculinisation in the polar bear (Wiig *et al*., 1998), reproductive impairment in the European otter (*lutra lutra*) in the UK and Eire (Mason, 1995), reduced testosterone levels in Dall's porpoises (*Phocenoides dalli*) in the Pacific ocean (Subramaniam *et al*., 1987) and decreased fecundity in harbour seals from the Wadden Sea (Reijnders, 1986), all associated with PCB contamination (congeners not always identified). Non-reproductive effects suspected to be linked to PCBs include skull lesions in harbour seals from the Baltic Sea (Olsson *et al*., 1994), reduced levels of vitamin A and thyroid hormones in the common seal (Brouwer *et al*., 1989), adrenocortical hyperplasia in grey and ringed seals from the Baltic Sea (Bergman and Olsson, 1995) and lowered immuno-competence in harbour seals from the Wadden Sea (De Swart *et al*., 1996).

A lot of attention has focused on the reduction of Baltic grey and ringed seal populations, and there is much evidence to show that populations have declined noticeably over the past century (Vos *et al*., 2000, and references therein). Evidence shows that mammals have been adversely affected by EDCs; however, limited field evidence exists to support the contention that the effects are through some endocrine-dependent mechanism. In order to understand the mechanisms by which EDCs affect mammals, knowledge of their endocrinology is needed and is a basic research requirement that once established, will enable better protection of mammals.

Fossi and Marsili (2003) state that future research needs should include the development of new methods to study ED, specifically non-destructive techniques that can replace lethal approaches for hazard assessment and the conservation of endangered species of aquatic mammals. This is an area where a molecular approach would be beneficial. Tabuchi *et al.* (2006) examined the feasibility of developing TR gene expression in free-ranging harbour seals (*Phoca vitulina*) as a biomarker of exposure to PCB-related compounds using skin/blubber biopsies. Using a biopsy-based approach, they were able to quantify the expression of TR genes in blubber. There was a positive correlation between TRα expression and PCB concentrations in harbour seals. Mos *et al.* (2007) have since used the technique on harbour seals to characterise the retinoic acid recpetor α (RARα) and investigate its expression levels along with vitamin A concentration as a potential biomarker of PCB exposure in marine mammals. A biopsy approach was used when it was first demonstrated that an aryl hydrocarbon receptor agonist (β-naphthoflavone) could induce CYP1A1 in whale tissue in a concentration dependent manner (Godard *et al*. 2004). The biopsy technique combined with a molecular approach could be applied to other aquatic mammalian species using other potential biomarkers as a minimally invasive mechanistic method of determining EDC exposure on endangered or protected species.

4.3 Birds

Birds have been exposed to a number of persistent organic chemicals. Fish-eating birds in particular are top predators in the aquatic food chain, and are thus vulnerable to the effects of persistent chemicals that accumulate as they move through the food chain. Much evidence has been gathered which indicates that fish-eating birds have been suffering from contaminants affecting reproduction as a result of exposure to polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), coplanar PCBs (PCB congeners that can assume a coplanar configuration), PAHs and the persistent pesticide DDT and its degradation product DDE. Effects of exposure to persistent organic chemicals on birds have been seen in a wide range of species including eagles, hawks, gulls, cormorants, herons, terns and more. Fry, (1995), CSTEE, (1999), Giesy *et al.* (2003) and Ottinger *et al*. (2005) provide overviews of studies conducted on suspected EDCs in birds and the consequences of effects on reproductive outcomes.

Briefly, reproductive effects are seen in both embryos and adult birds. In embryos, impairment of reproduction is characterised by high embryonic and chick mortality or reduced hatchability; failure of chicks to thrive (wasting syndrome); skeletal abnormalities; and impaired differentiation of the reproductive and nervous systems through EDCs mimicking oestrogens. Fish-eating birds nesting in the Great Lakes basin have been characterised by a syndrome known as GLEMEDS (Great Lakes embryo mortality, edema and deformities syndrome). Effects in adult birds include reduced fertility, suppression of egg formation, behavioural abnormalities, sex ratio skew and female-female pairing and the infamous effects of DDE on egg shell thinning. Egg shell thinning is correlated with DDE inhibition of shell gland calcium ATPase and

the species most susceptible appear to have a reduced ability to metabolise organochlorines (see Fry, 1995 and references therein). However, research shows that egg shell thinning is a complex phenomenon that could be attributed to a variety of environmental factors (Giesy *et al*., 2003).

Linking suspected EDC exposure to the exact mechanism leading to reproductive disorders is a largely unexplored area in birds. A potential mechanism of action that has been addressed has been the AhR-mediated induction of cytochrome P450 (CYP) enzymes. AhR is a ligand-activated transcription factor involved in the regulation of genes which include those for xenobiotic metabolising enzymes such as cytochrome P450 1A and 1B forms. Ligands for the AhR include a variety of aromatic hydrocarbons, including the chlorinated dioxins and related halogenated aromatic hydrocarbons whose toxicity occurs through activation of AhR. The mechanism by which CYP enzymes are potentially connected to altered sexual behaviour and reproductive failures is via a role in steroid metabolism (Bosveld and van den Berg, 2002). After reviewing results from studies assessing potential mechanisms of CYPs, Bosveld and van den Berg concluded that although effects of organochlorines on steroid metabolism have been shown, combined results do not support the hypothesis that alterations of the levels of circulating steroids by contaminant-induced (or inhibited) CYPs causes the observed effects on reproductive performance in environmentally exposed fish-eating birds.

4.3.1 Molecular analysis of CYPs, AhR and dioxin-related compounds

Recent molecular analysis of the induction of CYP1A and its relationship to dioxin and dioxin-related compounds has been conducted on the common cormorant (*Phalacrocorax carbo*), a species the Japanese Ministry of the Environment uses as a model organism to monitor environmental dioxin-related contaminants (Kubota *et al*., 2005, 2006; Nakayama *et al*., 2006). Kubota *et al*. (2006) cloned two CYP1A isoforms from the liver of cormorants from Lake Biwa, Japan. The isoforms were designated CYP1A4 and CYP1A5, based on their overall amino acid similarity to the chicken (*Gallus gallus*) CYP1A4 (78 per cent) and CYP1A5 (78 per cent). mRNA expression levels of both isoforms were positively correlated with total tetrachlorodibenzo-*p*-dioxin (TCDD) toxic equivalents and in most cases (with the exception of 2,3,7,8-tetrachlorodibenzofuran and 3,3',4,4'-tetrachlorobiphenyl (PCB77) which was proposed to be a result of rapid metabolism), to the concentrations of each congener. Liver-to-muscle ratios for most dioxin-related compounds increased with increasing induction of CYP1A4 and CYP1A5. The authors suggest that both CYP enzymes could be induced by a shared transcriptional mechanism, and that sequestration of some dioxin-related compounds in the liver of the cormorant occurs via binding to CYP genes. Kubota *et al*. (2005) assessed CYP1A proteins in the cormorant, where the proteins showed positive correlations with TCDD toxic equivalents. The combined studies showed that dioxin-related compounds induce CYP1As in the liver at both the transcriptional and translational level.

Nakayama *et al.* (2006) used a microarray spotted with 1,061 ESTs from the liver of wild cormorants from Lake Biwa to screen contaminant responsive genes, predict potential toxic effects and understand their mechanisms at the molecular level. To determine contaminant responsive genes, the relationship between contaminant concentration (polychlorinated dibenzo-*p*-dioxins, furans, polychlorinated biphenyls, 1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl) ethane and its metabolites (DDTs), hexachlorocyclohexane isomers, chlordane compounds, butylins and bisphenol A) and mRNA expression levels were examined using multiple regression.

Analysis of the microarray data was further quantified by real-time PCR. Significant correlations of genes relating to xenobiotic-metabolising enzymes (CYPs), receptors, immune function, antioxidants and glycoproteins were established with liver contaminants. Positive and negative correlations were detected in 66 and 70 genes respectively out of the 1,061. Two-thirds of genes whose expression was confirmed by real-time PCR significantly correlated with expression levels from the microarray. The authors suggest that those genes that did not correlate between the two methods may have been a result of cross hybridisation to non-target genes. This highlights the importance of using real-time PCR to confirm microarray results that have been used for screening responsive genes. Comparable to the results of Kubota *et al.,* TCDD toxic equivalents were positively correlated with CYP1A gene expression. In contrast, the antioxidant enzyme, Cu/Zn SOD, was negatively correlated which may indicate a chemically induced role in oxidative stress.

Karchner *et al*. (2006) assessed the molecular basis of dioxin sensitivity and the role of the AhR in the common tern (*Sterna hirundo*), an aquatic bird highly exposed to halogenated aromatic hydrocarbons, and the domestic chicken, the typical avian model. They cloned and sequenced AhRs from the tern and chicken and the full-length cDNAs encoded proteins of 859 amino acids and 858 amino acids respectively. Ligand binding studies showed that the common tern was less sensitive to halogenated aromatic hydrocarbons compared to the chicken, even though AhRs from both species exhibited specific binding of [^{3}H]TCDD. To assess the functional roles of the receptors, chimeric constructs of the AhRs were prepared. Chimeric constructs were made by swapping the functional domains (DNA binding/dimerisation, ligand binding and transactivation domains) of the receptors. The reduced ability of the tern AhR to bind to [^{3}H]TCDD *in vitro* and activate transcription was due to two amino acid differences in the ligand binding domain. Site-directed mutagenesis (which allows switching, insertion or deletion of single or multiple amino acids) confirmed that the reduced ability of the tern AhR to bind [^{3}H]TCDD was the result of Val-325 and Ala-381 amino acid residues in the ligand binding domain. Other avian species such as the common cormorant and black-footed albatross also have the Ala-381amino acid, whereas the chicken's is substituted for serine (Yasui *et al*., 2004).

Ramadoss and Perdew (2004) assessed the binding affinity of the human and mouse AhRs *in vitro* and found that the lower binding affinity of the human AhR compared to the mouse's was due to a different amino acid in the same position, Val-381. When they substituted the amino acid for Ala-381, high affinity binding of the human AhR to TCDD was restored. Structural modelling of the AhR infers that the amino acid residue at position 382 forms part of the antiparallel β-sheet in the ligand binding pocket of the receptor, and the reduced binding affinity of AhRs with a Val residue is the result of a larger side-chain as compared to Ala, which hinders access to the binding cavity (Karchner *et al*., 2006, and references therein). The authors showed that the presence of a Ser in the chicken appears to enhance the binding affinity and/or capacity, potentially by stabilising the receptor interaction through hydrogen bonding interactions with the dioxygen bridge of TCDD.

Studies like this provide a molecular understanding of the differences in sensitivity of avian species to environmental contaminants, and are essential for generating the mechanistic information required to extrapolate among species for risk assessment.

4.3.2 The chicken genome

Many advances in avian endocrinology have been made possible through the development of new research tools. Sequencing of genomes is an obvious one and the chicken is the first avian model organism, as well as the first agricultural animal, to

have its genome sequenced and analysed. Chickens have been involved in elucidating the molecular basis of limb development and cell migration in the nervous system, and have been used to elucidate the molecular basis of many limb and neural defects in humans. The International Chicken Genome Sequencing Consortium has analysed the chicken genome and compared it to the human, mouse, rat and puffer fish genomes (International Chicken Genome Sequencing Consortium, 2004). It has been estimated that approximately 60 per cent of chicken genes correspond to a similar human gene and as a result, will help us understand the structure and function of human genes. The analyses showed that genes that were generally conserved between the chicken and human genomes were often conserved in fish and were more likely to be present in all vertebrates.

The number of ESTs generated from the chicken has noticeably grown in recent years and includes:

- BBSRC ChickEST Database (http://chick.umist.ac.uk/) provides access to 339,314 ESTs generated from 21 embryonic and adult tissues.
- The University of Delaware ChickEST database (http://www.chickest.udel.edu/) contains over 40,000 ESTs from chicken cDNA libraries including those from the liver, oviduct, pituitary, reproductive tract and muscle and immune system.

ESTs are important in providing the basis for microarrays. A chicken multi-tissue cDNA microarray containing 13,000 features and covering 24 different tissues or cell types is now available to academics (Burnside *et al*., 2005). This tool will have use in developmental biology from an endocrinological perspective and is likely to enhance the chicken as a biological avian model.

4.4 Amphibians

Amphibians consist of three groups of vertebrates: frogs, salamanders and caecilians (found in the tropics only). Most of the research into endocrine disruption on amphibians has been conducted on frogs and toads (members of the order Anura). There are over 5,000 species of frogs and toads worldwide. Amphibians breathe partially (some species completely) through their skin and as a result are constantly exposed to the environment, making them more vulnerable to chemical contaminants. Populations of frogs and toads have been reported to be in decline in both polluted and pristine habitats around the world (Vos *et al*., 2000). Recent reports on amphibians suggest that 32 per cent of known species are globally threatened, higher than for birds and mammals (Baillie *et al*., 2004). Despite these declines, in many cases evidence is not conclusive in linking the stressor to population declines in the field. Frogs and toads inhabit both terrestrial and aquatic niches and are therefore exposed to different environmental stressors at different stages in their life cycle, making it more difficult to link the cause and effect of toxicant exposure.

The concern over declining populations has led to many hypotheses to try to explain such declines. These include exposure to UV-B radiation (280–320 nm), disease, habitat destruction, introduction of exotic species, global climate change and environmental contamination. The selective herbicide atrazine and other potential EDCs have been considered. It is, however, unlikely that any single factor is responsible for the observed declines.

EDCs could contribute to changes in amphibian population levels via adverse effects on reproduction and the thyroid system. In amphibians, they can affect reproduction by (anti-)oestrogenic and (anti-)androgenic modes of action that produce severe effects, including abnormal sexual differentiation (Kloas, 2002). Actions on the thyroid system cause acceleration or retardation of metamorphosis (Kloas, 2002). It has been proposed that amphibians provide suitable models to study the effects of ED,

especially effects on the thyroid system, since effects triggering metamorphosis can be determined easily and sensitively in amphibians compared to other vertebrates (Kloas, 2002), and also because there is a high level of evolutionary conservation of the thyroid system among vertebrates.

4.4.1 Reproductive abnormalities

Several authors have reported effects of EDCs on gonadal differentiation. Kloas *et al.* (1999) reported that bisphenol A and 4-nonylphenol significantly altered sex ratios in *Xenopus laevis*. However, these findings have been questioned by Staples *et al*. (2002). Kloas *et al.* (1999) used concentrations of 0, 2.3 and 23 μg/L BPA, however, Staples *et al*. (2002) reports that the concentrations were not analytically verified and the tests were not conducted under good laboratory practice (GLP), compared to a study by Pickford *et al.* (2000) which was conducted under GLP, where no skewing of sex ratios was observed at concentrations ranging from 0.1 to 500 μg/L BPA. Ohtani *et al*. (2000) exposed male tadpoles of *Rana rugosa* to dilute solutions of a widely used plasticiser of polyvinyl chloride resins, dibutyl phthalate (DBP), during days 19-23 after fertilisation (critical period of gonadal sex differentiation). They observed induction of ovarian tissue in 17 per cent of tadpoles at 10 μM DBP (approximately 1,000-fold lower than that observed with 17β-oestradiol). Qin *et al*. (2003) reported effects on gonadal differentiation in *Xeonopus laevis* after exposure to 100 μg/L PCB from Nieuwkoop and Faber stage 46/47 to complete metamorphosis (lower environmentally relevant concentrations did not elicit a toxic response after 10 days exposure). The chemical herbicide atrazine has been shown to demasculinise male frogs at environmentally relevant concentrations through oestrogenic or anti-androgenic mechanisms (Hayes *et al*., 2002, 2003).

Research on the effects of atrazine exposure has been somewhat controversial since Professor Tyrone Hayes published his results in 2002, which showed that one of the most widely used (second to glyphosate) herbicides could feminise male frogs at concentrations thirty times lower than the legally allowed limit of three micrograms per litre in US drinking water (Hayes *et al*., 2002). These effects could have severe consequences on the ability of frog populations to reproduce. In addition, there is the potential for atrazine to affect human health via the same mechanism of action. The study exposed *X. laevis* larvae to a range of atrazine concentrations (0.01 to 200 μg/L). Gonadal histology and larynge size at metamorphosis were examined. At atrazine concentrations greater than 0.1 μg/L, hermaphroditism and demasculinisation of the larynges were observed. The frogs typically had both ovaries and testes and had smaller voice boxes (important for making the mating call). Hayes *et al*. (2003) subsequently demonstrated hermaphroditism at the same concentrations in *Rana pipiens*. It has been hypothesised that atrazine exposure induces P450 aromatase, the enzyme that converts androgens to oestrogens, and this mechanism has been confirmed in fish, amphibians, reptiles, birds, mammals and humans (Hayes, 2005 and references therein).

These findings sparked a critical response from atrazine's manufacturer Syngenta Crop Protection and Syngenta-funded scientists. However, data presented to the US EPA failed to provide conclusive evidence that there are no adverse effects from atrazine exposure. Hayes (2004) has further stated that data presented to the EPA suffered from contaminated laboratory controls, high mortality, inappropriate measurements of hormone levels, sexually immature animals and contaminated reference sites. The controversy continues.

Despite the uncertainties surrounding the effects of atrazine exposure, the US EPA approved its continued use in October 2003, the same month that the European Union (EU) announced its ban due to its ubiquitous and persistent nature.

Hayes' results and the consequences of low-dose exposure have not been widely accepted because they cannot be reproduced in other laboratories (Coady *et al*., 2005). Many of the studies on atrazine have been performed at concentrations much higher than those used by Hayes. However, including the work by Hayes *et al.* (2002, 2003), effects have been implicated for exposures of 0.1 to 25 μg/L (many times greater than what frogs could be exposed to in the field), with induced gonadal abnormalities and hermaphroditism in *Rana pipiens* and *Xenopus laevis* (Tavera-Mendoza *et al.,* 2002; Carr *et al*., 2003), supporting the conclusion that atrazine is a potent endocrine disrupter.

It appears that the atrazine controversy would benefit from a molecular approach to determine its mechanism of action. Hecker *et al.* (2005) have tried to address this issue using Q-PCR *CYP19* (a gene shown to be upregulated by atrazine in a human adrenocarcinoma cell line (Sanderson *et al*., 2000)) gene expression and aromatase activity on plasma sex steroid concentrations in *X. laevis*. They report that in a series of studies conducted on atrazine to investigate its mechanism of action, none showed any significant effect on aromatase *in vitro* in a rat cell line or *in vivo* in juvenile fish, developing and adult frogs and a juvenile reptile (see Hecker *et al*., 2005, and references therein). However, because steroid regulatory mechanisms among different species, development stages and between genders differ in response, a direct comparison to Hayes's work is not possible. Therefore, the study by Hecker *et al*. was designed specifically to analyse the mechanism of atrazine proposed by Hayes *et al*. (2002), and use multiple lines of evidence to measure the effects on oestrogen synthesis. *X. laevis* frogs were exposed to 1, 25 and 250 μg/L atrazine for 36 days. Both *CYP19* and aromatase activity were low regardless of treatment, and the authors concluded that the tested concentration did not interfere with steroidogenesis through an aromatase-mediated mechanism of action.

4.4.2 Thyroid disruption

Amphibian metamorphosis has been used as a model to investigate the development function and mechanism of gene regulation by TRs, and also to understand how EDCs affect thyroid function. To understand the development role of TR, it is important to understand the molecular basis by which TR mediates its effect of T_3 in various processes (Buchholz *et al*., 2003). The importance of the thyroid axis in inducing metamorphosis has been known since the early 1900s, when it was demonstrated that TH is responsible for inducing amphibian metamorphosis (OECD, 2004). For a review of amphibian metamorphosis and the role of endocrine systems, see Shi (2000) and Denver *et al*. (2002).

Frog metamorphosis involves the complex transformation of every organ and tissue of the tadpole, where organs and tissues undergo vastly different changes. Despite these differences, all are controlled by T_3 (OECD, 2004). Metamorphosis has three phases: 1) premetamorphosis, where the tadpole is competent to respond to exogenous TH (which can induce precocious or early metamorphosis); 2) prometamorphosis, which begins with maturation of the thyroid gland and low-level secretion of TH that initiates the first metamorphic changes such as limb growth; and 3) metamorphic climax, characterised by rapid, overt remodelling of the tadpole (fore limb development and tail resorption) and a peak in TH (Zhang *et al*., 2006).

Tissue responsiveness to THs is based on a mechanism that involves hormone binding to TR, initiating a tissue-specific activation or repression of target genes. In amphibians, studies have revealed a dual role for TRs (see review by Sachs *et al.*, 2000). Initially, TRs act as repressors of TH-inducible genes in premetamorphic tadpoles, to prevent precocious metamorphosis and ensure proper tadpole growth, and then they become activators of these genes, to activate the metamorphic process. Their second role is to promote cell proliferation and apoptosis during metamorphosis, which is dependent upon the cell type they were expressed in.

Since frog metamorphosis is a well-studied phenomenon driven by the thyroid, and offers a representative vertebrate model for evaluating the effects of EDCs on the thyroid system, an amphibian metamorphosis assay has been proposed by the Organisation for Economic Cooperation and Development (OECD) Task Force on Endocrine Disrupters Testing and Assessment, for identifying substances with the potential to disrupt the thyroid system (OECD detailed review paper, 2004). The paper describes the state-of-the-knowledge on amphibian metamorphosis, provides the scientific basis of the assay and describes candidate endpoints reflective of thyroid dysfunction. It is proposed as a short-term test with morphological, biochemical and molecular-based elements.

Three molecular approaches are currently being evaluated. These are single gene expression assays, multiple gene expression arrays (microarrays), and transgenesis of relevant reporter gene constructs. There is growing interest in the development of molecular endpoints, such as measuring rapid changes in gene expression, and Sections 3.4.2.1 – 3.4.2.3 describe research which has applied molecular approaches to generate information on the mechanism of action of EDCs on metamorphosis.

4.4.2.1 Single gene expression assays

Zhang *et al.* (2006) point to the need to develop additional tools, given that standard toxicological approaches which focus on tissue and organism effects are unable to adequately discern molecular mechanisms of action. Gene expression monitoring can potentially serve as a reliable predictor of whole organism effects while providing crucial information on mechanisms of action of a particular chemical, class of chemicals or mixtures of unrelated chemicals (Zhang *et al*., 2006). Since gene expression changes often precede overt morphological and physiological changes, development of molecular endpoints for incorporation into existing assays could reduce assay duration (Crump *et al*., 2002; Zhang *et al*., 2006) and allow for greater predictive capability and earlier assessment of EDC action in wildlife species (Veldhoen and Helbing, 2005).

Gene expression assays provide mechanistic information on chemicals that affect specific genes during metamorphosis. Many of the genes involved in the activation or repression of metamorphosis have been identified in *X. laevis* and the early response gene TRβ appears to be essential, according to Yaoita *et al.* (1990). They found a close correlation between the expression of TRβ mRNA with metamorphosis and the upregulation by TH, reaching maximal levels at the climax of metamorphosis when endogenous TH is maximal. TRα levels were only minimally affected. The TRβ gene could thus be selectively monitored for activity during metamorphosis and used as a molecular biomarker.

Veldhoen and Helbing (2001) assessed gene expression in *Rana catesbeiana* using a tail fin biopsy from live premetamorphic tadpoles. *R. catesbeiana* premetamorphic tadpoles were exposed to 100 nM T_3 to induce metamorphosis, and significant

elevation of TRβ mRNA levels was recorded within 24 hours. Expression was significantly enhanced upon subsequent exposure to10 nM acetochlor, a herbicide previously shown to accelerate TH-induced metamorphosis. To the authors' knowledge, this was the first demonstration of perturbation of a key TH-dependent gene by an EDC. The authors suggest that the method used for evaluating exposure to EDCs could potentially be used in field studies in different species.

Brominated phenolic and phenol compounds have been known to target transthyretin receptors in plasma, TH metabolism and TR-regulated gene expression. There are only a few environmental chemicals that compete with T_3 binding at concentrations below 10^{-6} M, suggesting that sites other than T_3 binding are their main targets (Kudo *et al.,* 2006, and references therein). Kudo *et al.* (2006) investigated the *in vitro* (competitive binding) and *in vivo* (short-term gene expression assay using real-time PCR) effects of brominated phenolic and phenol compounds on the thyroid system. They assessed the binding of the hormone ^{125} I-3,3',5-L-triiodothyronine (^{125}I-T_3) to purified *X. laevis* transthyretin (xTTR) and to the LBD of the thyroid hormone receptor β (xTR LBD) on the induction of a T_3-responsive reporter gene in a recombinant *X. laevis* cell line (XL 58-TRE-Luc) and on T_3-induced or spontaneous metamorphosis in *X. laevis* tadpoles. Structures with a bromine in either *ortho* position with respect to the hydroxyl group competed more efficiently with T_3 binding to the xTTR and XTR LBD. 3,3',5-tribromobisphenol A (brominated derivative of bisphenol A) at submicromolar (5 μM) concentrations was shown to exert both an agonistic and antagonistic effect in the *in vitro* reporter gene assay, which was confirmed in the *in vivo* short-term gene expression assay using premetamorphic *X. laevis* tadpoles and endogenous T_3-responsive genes as molecular markers. The *in vivo* studies were not as clear as the *in vitro* cell line study. In addition, the thyroid disrupting activity of 3,3',5-tribromobisphenol A was barely detected in the trunk and was not detected in the head, indicating the sensitivity of different tissues that must be considered when investigating different endpoints. It was suggested that 3,3',5-tribromobisphenol A affects binding of T_3 to xTTR and TR, and that it interferes with intracellular T_3 signalling pathways.

Pharmaceuticals and personal care products (PPCPs) present a source of potential EDCs in the environment. The bactericidal agent triclosan (TSC) is of concern because it is structurally very similar to TH. Overt toxicity of TCS exposure on young tadpoles (concentrations up to 230 μg/L) have been demonstrated on different frog species, but the potential sublethal effects, such as disruption of TH-mediated action in the context of metamorphosis, have not, according to Veldhoen *et al.* (2006). Using Q-PCR, the authors showed that exposure of *R. catesbeiana* to TSC as low as 0.03 μg/L disrupts thyroid hormone-associated gene expression; it also produces decreased levels of TRβ mRNA in the tadpole tail and increased levels of proliferating nuclear cell antigen transcript (codes for a protein that is one of the central molecules responsible for decisions of life and death of the cell) in the brain of premetamorphic tadpoles.

4.4.2.2 Multiple gene expression arrays

Crump *et al.* (2002) developed a 420 gene cDNA array (multi-species analysis of gene expression MAGEX array) derived from known frog sequences (390 sequences from *X. laevis* and 30 from *R. catesbeiana* reflecting their abundance in GenBank) and used the array to determine the effects of 10 nM acetochlor on premetamorphic X *laevis* tadpoles. Tadpoles were exposed to 10 nM acetochlor in the presence (during precocious metamorphosis) and absence of 100 nM T_3 for up to 72 hours. Morphometric analysis was also conducted after exposure at 48 and 72 hours.

Morphometric analysis showed no significant effect on body area after exposure to all treatments at 48 hours. After 72 hours, tadpoles exposed to acetochlor alone showed a modest decrease in body area, but results were statistically insignificant. However,

exposure to T_3 resulted in a significant ($p < 0.05$) reduction in body area and the adoption of adult morphology in the head region compared to the control. This effect was more pronounced in the combined treatment with acetochlor and T_3 causing again a significant ($p < 0.01$) reduction in body size, but with more extensive sculpturing of the head, reduced fin quality and increased pigmentation, suggesting the additive effect of acetochlor accelerating the metamorphic process.

Using the MAGEX array, 11 genes were altered in the tail tissue upon exposure to acetochlor alone within 48 hours after exposure. They included genes encoding transcription factors, signalling molecules and apoptotic proteins. All genes except the GSK-3 binding protein (which was upregulated) were downregulated in relation to the control.

In response to T_3 treatment alone and in combination, 26 genes displayed altered gene expression and were divided into three groups. Group one (10 genes) was upregulated by two-fold in response to T_3, which was further increased (with the exception of two genes) when combined with acetochlor treatment. Eight out of the 10 genes had previously been identified as thyroid hormone responsive genes. Group two genes displayed little or no response to T_3 but when combined, resulted in elevation of mRNA of all genes. Group three genes were downregulated in relation to T_3, but the addition of acetochlor attenuated the repression of gene expression. Finally, eight genes were identified that were not affected by acetochlor, but were responsive to T_3 during precocious metamorphosis.

In addition to using the array, Q-PCR was used to examine the expression levels of TRα and TRβ transcripts. Exposure to T_3 produced no significant change in TRα levels, but a significant ($p < 0.01$) five-fold increase in TRβ levels. Both TRα and TRβ transcript levels were not affected by acetochlor alone, but in combination a significant ($p < 0.05$) increase in TRα was detected relative to the control, with a further significant ($p < 0.01$) increase in TRβ levels.

Crump *et al.* (2002) established that acetochlor accelerates T_3-induced metamorphosis within 72 hours, and that alterations in gene expression can be detected before overt morphological changes. The alterations in gene expression profiles suggest a novel mechanism of action for acetochlor in *X. laevis*.

Helbing *et al.* (2003) and Veldhoen *et al.* (2002, 2006) have since used the MAGEX array to detect thyroid hormone responses in *X. laevis* and *Rana catesbeiana* tail tissues. Veldhoen *et al.* (2002) investigated temporal gene expression profiles in *X. laevis*. They monitored gene expression from premetamorphosis through to metamorphic climax using the array and Q-PCR. Seventy nine genes, 34 of which had not previously been described, were found to have altered mRNA steady-state expression levels during natural metamorphosis. Of the 79 genes altered, 28 were altered during premetamorphosis, 31 in prometamorphosis and 43 with the onset of tail regression.

Helbing *et al.* (2003) profiled T_3 hormone responsive genes and proteins in the tail of *X. laevis* tadpoles undergoing precocious metamorphosis, using the cDNA array combined with 2D gel electrophoresis and peptide mass mapping using mass spectrometry. They identified 93 T_3 responsive genes in the tail of premetamorphic tadpoles after exposure to exogenous T_3, 53 having not previously been characterised. In addition, several T_3 responsive proteins were identified. These included tubulin isoforms and ribonucleotide reductase protein R1. Ribonucleotide reductase protein R1 has been shown to bind to γ and α/β tubulin *in vitro* and promote microtubule nucleation on the centrosome at the onset of mitosis in *Xenopus* egg mitotic extracts. Other proteins isolated included desmin, an intermediate filament protein whose mRNA increases during embryogenesis, and creatine kinase, an enzyme important in energy

metabolism (Helbing *et al.,* 2003, and references therein). Veldhoen *et al*. (2006) applied the MAGEX array to *Rana catesbeiana* in an attempt to extend gene analysis expression to other more relevant indigenous species. A comparison of the two arrays revealed that the response to T_3 treatment within tail tissue for both species was predominantly upregulated in TH responsive mRNA transcript levels 48 hours after administration of the hormone. Using cDNA microarrays across species can help identify evolutionary conserved gene expression programmes, along with suitable biomarkers for screening EDCs.

Veldhoen *et al.* (2006) noted that progress in determining the ED effects of chemicals in amphibians using a cDNA microarray has been impeded by the paucity of genomic information on native species (non-model organisms). The coverage of ranid sequences represents only seven per cent of the MAGEX array. In addition, most of the ranid sequences in GenBank are in the form of expressed sequence tags. This highlights a need to generate sequence information on non-model organisms.

X. laevis has been the preferred model organism representing amphibians to investigate vertebrate embryonic development, essentially because it is amenable to laboratory manipulation and its rapid embryonic development cycle makes it powerful to understand gene and proteins functions (Koide *et al.,* 2005). There is, however, wide acceptance that this organism is not ecolologically representative for many parts of the world. In addition it has undergone a genome-wide duplication, meaning that most genes are represented by two paralogues, which will have implications for the construction of microarrays in that there will be less coverage of a similar microarray from a closely related species (Chalmers *et al.,* 2005). More recently, focus has been on *X. tropicalis* (now *Silurana tropicalis*) which is diploid and therefore better for genetic studies. In addition, it has a small genome size and generation time (four months). The genome sequence of *S. tropicalis* has now been completed (http://genome.jgi-psf.org/Xentr4/Xentr4.home.html) and will enable the study of gene activation at the genome wide level and allow target genes affected by EDCs to be identified. Two *Xenopus* gene chips are now commercially available: the *X. laevis* GeneChip® from Affymetrix that can be used to study the expression of over 14,400 gene transcripts, and the Array-Ready Oligo Set™ with over 10,000 70mer probes on the chip from *S. tropicalis* and made by Operon Biotechnologies, Inc. Chalmers *et al.* (2005) produced a pilot cDNA array based on *S. tropicalis* sequences to test if it would work across species on *X. laevis*. They analysed genes whose expression levels changed during early development of both species. The experiments gave similar results and the authors suggest that researchers can still work with *X. laevis,* but make use of the advantages offered by *S. tropicalis* microarrays. There is an obvious need for comparative studies to confirm if *X. laevis* is a good surrogate for native species, and if results can be extrapolated across species.

4.4.2.3 Transgenesis

Gene manipulations have been used for a long time to study gene function. With vast amounts of information being generated through the use of microarray analyses, a major challenge ahead is to disentangle the function of thousands of genes. Gene knockouts and transgenic lines provide a physiological means of studying gene function. Kroll and Amaya (1996) offer an example of how to establish a transgenic frog line for functional genomic analyses on *Xenopus*. Essentially, linearised plasmid DNA is introduced into demembranated and decondensed *X. laevis* sperm nuclei *in vitro*, using restriction enzyme mediated integration (REMI). The nuclei are subsequently transplanted into fertilised eggs, yielding hundreds of normal diploid embryos per day which develop to advanced stages and express the integrated plasmids. Sparrow *et al.*

(2000) have since developed a simplified version which, although it bears similarities, does not require sperm decondensation or treatment with restriction enzymes, which can cause aneuploid embryos that do not develop properly. Their technique uses uncondensed sperm nuclei, reducing the incidence of aneuploid embryos and improving overall health. If transgenesis is combined with antisense oligonucleotides (AON), RNA inference (RNAi) or dominant-negative mutant approaches to suppress specific gene expression, then functional 'knockouts' of specific genes can be produced to determine loss of function of targeted genes.

For over a decade, Professor Barbara Demeneix's laboratory at the Muséum National d'Histoire Naturelle in France have been developing somatic gene transfer methods to assess gene regulation during amphibian metamorphosis (Luze *et al.,* 1993; Trudeau *et al.,* 2004). More recently, they have introduced germinal transgenesis originally developed in 1996 by Drs Kroll and Amaya (Turque *et al.,* 2005). Somatic gene transfer is a simpler method, whereby the exogenous gene is inserted (injected) directly into the tissue you want to study the expression in, and you can follow expression *in vivo* to obtain temporal and spatial expression data. Somatic transgenic experiments are smaller scale, and are ideal for comparing responses to different constructs and for setting up physiological constructs. There is no need to maintain frog lines, and many test situations can be compared simultaneously (Turque *et al.,* 2005). However, once the construct has been established and its functionality tested in a somatic transgene experiment, germinal transgenesis is much more efficient for scaling up for screening purposes, as several hundred tadpoles per brood can be produced (Turque *et al.,* 2005). In addition, germinally transgenic tadpoles can be used in long-term exposure experiments to test potential EDCs. The fluorescent signal provides information on tissue specificity and developmental stage during metamorphosis.

Turque *et al.* (2005) developed a rapid (shorter time frame than several weeks needed to record morphological changes), sensitive physiological *in vivo* method for testing the transcriptional effects of thyroid disrupting agents in premetamorphic *X. laevis* tadpoles, and applied the method to test the effects of acetochlor. They used somatic (in muscle and brain) and germinal methods to produce transgenic tadpoles with TH responsive elements (TH/bZIP promoter) coupled to either luciferase or green fluorescent protein, in order to follow T_3-dependent transcription *in vivo*. Using somatic gene transfer, they tested the transcriptional responses to TH agonists (TRIAC and T_3) after exposure for 48 hours. Tadpoles exposed to TRIAC displayed a three-fold increase in transcription from the TH/bZIP promoter compared to the control. They tested the sensitivity of the method in the muscle and brain, where the brain proved to be a more sensitive TH target than muscle in terms of the TH/bZIP-luc construct. The same construct was used in germinal transgenesis and fluorescence followed during early development and metamorphosis, focusing on the brain and limb buds. In limb buds, the transgene is barely expressed at NF stage 51 and remains weak until NF stage 61-62. During metamorphosis, the signal intensifies. The transgene is expressed in the central nervous system at NF stage 51 and the fluorescence signal increases gradually through each metamorphic stage. When used to assess the effects of 48 hour exposure to 10^{-10} M T_3 and 10^{-10} M T_3 plus 10^{-8} M acetochlor (lower than previous studies such as Crump *et al.,* 2002) on NF stage 52 tadpoles, there was a 20 per cent increase in fluorescence in the brains of germinally transgenic tadpoles, showing that transcriptional assay allows detection of thyroid disrupting activity at environmentally relevant concentrations.

Buchholz *et al.* (2003) used a dominant negative thyroid hormone receptor (dnTR), ubiquitously expressed in transgenic *X. laevis,* to study the molecular mechanism of TR function in T_3-induced metamorphosis *in vivo*. The receptors are essentially inactive and inhibit normal receptor function in a dominant negative manner to cause hormone

resistance. Transgene expression was confirmed by observation of fluorescence from a green fluorescent protein (GFP). The transgenic *X. laevis* tadpoles expressing the dnTR (TRα isoform) blocked T_3-induced metamorphosis at the beginning of prometamorphosis, and the dnTR inhibited the expression of known T_3 responsive genes. The study provided evidence that T_3-induced development requires TRE binding by TR, release of corepressors and histone acetylation for gene activation by T_3 and subsequent morphological changes. This supports the proposed model of a dual function role of TR in frog development (Sachs *et al.,* 2000), as an activator via the release of corepressors and promotion of histone acetylation and gene activation.

In an attempt to clarify the effects of BPA and related compounds on T_3-induced and spontaneous tadpole tail regression, Goto *et al.* (2006) produced transgenic *X. laevis* using the method of Kroll and Amaya (1996), with TRE of the TRβA 1 gene linked to a green fluorescent protein. BPA, TBBPA, TCBPA and TMBPA were found to inhibit T_3-induced tail regression in *Rana rugosa* as well as spontaneous metamorphosis by endogenous circulating TH in a dose-dependent manner using morphometric analysis, suggesting BPA and related compounds act as antagonists. Furthermore, exposure of T_3 to transgenic *X. laevis* tadpoles overexpressing TRβ induced the expression of the GFP gene. With subsequent exposure to BPA, TBBPA, TCBPA and TMBPA, expression was blocked in a dose-dependent manner. This suggests that binding of BPA and related compounds to the TR causes competition with T_3, resulting in suppression of TRβ-mediated gene transcription. Similar results were found for relatively low levels of BPA (10^{-7} M) using single gene expression analysis, where it was found to act as an antagonist of T_3 through the suppression of TRα and β (with moderate suppression of RXRγ) in *Xenopus* tail cultures (Iwamuro *et al.,* 2006).

TH regulates a wide range of biological processes during development, and since many EDCs have the potential to interfere with different aspects of the thyroid system function and TH action, this suggests a need to develop an *in vivo* assay for the detection of thyroid axis disrupting chemicals (Turque *et al.,* 2005). The authors point out that there is a long-standing debate in the field of endocrine disruption as to whether it is more important to reveal potential disrupting effects or to address the mechanisms of action underlying disruption. The protocol developed by Turque *et al.* (2005) will allow the *in vivo* detection of a wide of EDCs, rather than reveal mechanisms of action. Conversely, applying transgenesis with the dominant negative receptor, for instance (Buchholz *et al.,* 2003), reveals *in vivo* mechanistic information. Revealing both the potential ED effects of chemicals and mechanistic information is important, and the choice of transgenic method would depend on the question being asked. What is evident is that a transgenic approach which expresses a TH-dependent gene can potentially provide *in vivo* bioassays of TH-dependent gene expression, in a context where the mechanistic information can be linked to effects at higher levels of biological organisation, such as morphology (OECD, 2004).

4.5 Reptiles

Reptiles, like many other species, are potentially valuable indicators of ecosystem condition, as they are exposed to environmental contaminants. However, they have received very little attention regarding the effects of EDCs. Reptiles encompass a broad diversity of groups and include lizards, snakes, turtles, tortoises, crocodiles and alligators. Different life histories and reproductive modes including oviparity, ovoviviparity and viviparity, as well as both genetic and temperature-dependent (different in alligators to mammals) gender determination, are characteristic of many species.

Evidence suggests that developmental processes, particularly sex determination, gonad development, steroid hormone synthesis and development of secondary sex characteristics in reptiles, are susceptible to EDCs (Crain and Guillette, 1998 and references therein). The American alligator is probably the best studied reptilian example demonstrating endocrine disrupting effects, and research on this species has been increasing over recent years (Guillette and Iguchi, 2003). Reproductive disorders have been associated with alligators living in a pesticide-contaminated lake (Lake Apopka, Florida) in America (Crain and Guillette, 1998). Specifically, juvenile alligators in the lake presented abnormal gonadal morphology, altered steroidogenesis and changes in sex steroid concentration; male and female juvenile alligators had reduced testosterone and oestrogen plasma levels respectively. The majority of research on the alligator has been carried out by Guillette and colleagues and contaminant response data was reviewed in Guillette *et al.* (2000). More recently, the first reported study of abnormal bone composition (higher bone mineral density) was observed in female juvenile alligators in the same the lake (Lind *et al*, 2004).

In addition to alligators, studies have shown that snapping turtles (*Chelydra serpentine*) in the Great Lakes are also susceptible to developmental abnormalities (Bishop *et al.,* 1991, 1998). A total of 202 clutches from four different locations in the Great Lakes between 1986 and 1989 were analysed for hatching success, deformities and contaminant concentrations (Bishop *et al.,* 1991). Two of the sites containing the highest concentrations of organochlorines showed the highest incidence of deformities which included deformities of the tail (most common from all sites), hind legs, head, eyes, scutes, forelegs, along with dwarfism, yolk sac enlargement and missing claws. Hatching success was lowest in one of the two highly contaminated sites, while higher at the least contaminated site. PCBs were the chemicals most associated with deformities and hatching success, but other chemicals could not be excluded. From 1989 to 1991 at seven study locations in the Great Lakes, similar deformities were found and were shown to significantly increase with increasing concentrations of PAHs, particularly PCDD and PCDF. However, this was not correlated with the toxic equivalent factor (TEQs) in eggs (Bishop *et al.,* 1998). Sexual differentiation and reproduction have also been shown to be affected by organochlorine contamination in snapping turtles (de Solla *et al*., 1998, 2002). Alterations in secondary sexual characteristics occurred in early development, suggesting transfer of contaminants from exposed female to offspring (de Solla, 2002).

In the cases of both the alligators and the turtles, although the principal contaminants were identified, the actual link between chemical and observed effect was not established.

To begin to understand the molecular mechanism of hormone action in reptiles, the cDNA encoding hormone receptors, including the oestrogen, progesterone and thyroid receptors were recently isolated in different reptilian species (Katsu *et al.,* 2004, 2006; Helbing *et al.,* 2006). Sumida *et al.* (2001) had previously isolated full-length crocodile ERα sequences from *Caiman crocodiles* and *C. uniparens*.

Katsu *et al.* (2004, 2006) isolated full length ERα using 5' and 3' rapid amplification cDNA ends (RACE) from the ovary of the American alligator (*Alligator mississippiensis*) and the Nile crocodile (*Crocodylus niloticus*) respectively, and partial ERβ and PR cDNA fragments from the alligator. The Nile crocodile ERα showed high amino acid identity to the alligator (ERα 98%), caiman (ER 98%), lizard (ER 82%) and the chicken (ERα 92%). The alligator ERα amino acid sequence was 91% similar to the chicken. The authors also studied transcript expression using Q-PCR and in both reptiles, ERα was expressed in the ovaries and testes. In juvenile alligators injected with a single dose of 17β-oestradiol (270 μg/kg), ERα mRNA expression was reduced in the ovary within 30 hours of treatment, whereas ERβ and PR were unaffected. The authors tried

to isolate the cDNA clone of the Nile crocodile ERβ, however attempts were unsuccessful.

No reptilian TR sequences had been reported until the study conducted by Helbing *et al.* (2006). In addition, there had been no direct evidence for the regulation of reptilian TR gene expression by the thyroid axis. In their study, the authors isolated two distinct TRα mRNA transcripts (variants TRαa and TRαb) and one TRβ transcript from the American alligator (*Alligator mississippiensis*). TRαa and TRαb transcripts were 1404 and 1362 bp respectively. Neither sequence had an in-frame stop codon in the putative 5' untranslated region. They differed in the first 135 bp, with only 40 per cent identity in the overlapping 104 bp region. From that point on, they were identical.

Both TRαa and TRαb give rise to the same putative protein (~41 kDa), which is smaller than the protein (~45 kDa) derived from the chicken and human TRα, but displays 90% and 85% identity respectively despite the putative truncation. The alligator TRβ sequence is 99% and 95% identical to the chicken and frog respectively. Both TRα and TRβ are highly conserved between reptiles and other vertebrates, with the exception of the N-terminal end of the putative proteins. Steady-state levels of TR transcripts were measured in the heart, lung, thyroid, liver, cliterophallus/phallus and gonad of juvenile alligators 24 and 48 hours after injection with thyroid stimulating hormone (TSH). TRα transcript levels were increased in the heart, decreased in the lung and cliterophallus/phallus, and unaffected in the liver, thyroid and gonad. TRβ expression levels were an order of magnitude lower in all tissues compared to TRα, with highest levels observed in the heart, lung and cliterophallus/phallus. No change was observed in the liver, speculated to be of less importance in the functional role in this tissue. No significant differences of TR expression levels were detected between sexes. Administration of TSH resulted in tissue-specific responses, with the heart and lung being most sensitive.

Guillette *et al.* (2002) provide evidence that nine pesticide compounds found in the plasma of juvenile alligators exhibit a high affinity for the alligator ER using competitive binding studies (the most effective compounds being heptachlor epoxide and heptachlor). The study suggests that the compounds are capable of acting as ER agonists or antagonists; however, it doesn't provide direct evidence of their action *in vivo* and their mechanisms of action are still unclear. The recent isolation of oestrogen, progesterone and thyroid receptors will allow *in vivo* studies on the mechanistic action of potential EDCs in reptiles.

At present, reptiles are the only major vertebrate lineage for which there is still no comlpete genome sequence available. In July 2005, an *ad hoc* reptilian genomics working group submitted a proposal on behalf of a large international community of researchers to the National Human Genome Research Institute (NHGRI), to sequence the first reptilian genome of the green anole lizard (*Anolis carolinensis*) as well as the genome of the American alligator. In response, the NHGRI have selected the green anole lizard for the next round of sequencing and targeted it as priority for "high-quality draft" sequencing. Once sequenced, it will be the first reptile to have its genome sequenced and will provide valuable comparative information with the chicken, human and other mammalian genomes. The lizard is also a well established model for nerobiology, reproduction and endocrinology. Much research has been conducted on the role of steroid hormones in addition to their metabolising enzymes in the display of reproductive behaviors, and the same gonadal hormones that facilitate aggressive and reproductive behaviours in other vertebrate species activate these behaviours in anoles (Lovern *et al.,* 2004).

Several thousand ESTs from the cDNA library of adult alligators, and those from the gonads of embryos incubated at temperatures that produce all males or all females,

have been sequenced and clustered (Iguchi *et al.,* 2006). An alligator microarray is currently being established that will allow the study of the molecular mechanism of sex determination, chemical effects on sex determination and the toxic effects of chemicals.

4.6 Invertebrates

Invertebrates comprise most of the animals on Earth, more than 95 per cent to be exact. The phyla are incredibly diverse, ranging from simple sponges to insects to molluscs and worms. Arthropods are the most abundant and largest group of invertebrates, while molluscs are considered the most diverse group (deFur, 2004). Compared to vertebrate systems, only a limited number of studies have explored the effects of ED in invertebrates, largely due to a lack of knowledge on basic endocrine physiology of most invertebrate groups. For comprehensive reviews on endocrine disruption and invertebrates, see Pinder *et al.* (1999) and Roast and Benstead (2006). To illustrate the range of effects linked to EDCs in invertebrates, Table 3.1, taken from deFur (2004, see references therein), presents recent examples of invertebrate endocrine disruption research and includes ED compounds (which currently serve as reference standards), the species affected and the functions disrupted. The research areas cover insect moulting and development, molluscan sexual development and crustacean development.

Table 3.1: Observed effects of endocrine disrupting chemicals seen in invertebrates

Compound	**Animal**	**Effect**	**Reference**
Bisphenol A	Snails *Marisa cornuarietis* *Nucella lapillius*	Impaired reproductive organ growth	Oehlmann *et al*. 2000; 2005
Octylphenol	Snails *Marisa cornuarietis* *Nucella lapillius*	Impaired/enhanced reproductive organ growth	Oehlmann *et al*. 2000
Nonylphenol	Oyster *Crassostrea gigas* *Daphnia magna*	Feminisation; altered sex ratios	Nice *et al*. 2003 LeBlanc *et al*. 2000
Tributyl tin	Abalone *Haliotis gigantea*	Masculinisation of females	Horiguchi *et al*. 2002
Endosulfan	Grass shrimp *Palaemonetes pugio*	Acute mortality; delayed embryo hatch	Wirth *et al*. 2001
Methoprene	Grass shrimp *Palaemonetes pugio*	Acute mortality; delayed embryo hatch; altered growth	Wirth *et al*. 2001
Fipronil	Copepod *Amphiascus tenuiremis*	Male infertility	McKenney and Celestial, 1993 Chandler *et al*. 2004

Invertebrates have been shown to display comparable responses to EDCs with those observed in vertebrates; in some cases, snails may be more responsive to low concentrations of some xenestrogens than fish (Jobling *et al.,* 2004). If gastropods are found to be more responsive, then invertebrates could be considered as surrogate test organisms for vertebrates and replace fish testing, which is an area that should be explored in the interest of animal ethics. However, there is a fundamental lack of knowledge on invertebrate endocrinology and how the molecular mechanisms of EDCs link to physiological responses. Therefore, in order for regulators to ensure that adequate protection targets are in place, an understanding of invertebrates' basic endocrinology, their responses to EDCs and the underlying mechanisms in invertebrates requires investigation.

4.6.1 Molecular approaches unveil non-steroid nuclear receptors in molluscs

Despite the fact that research on invertebrates and ED has been done on a much smaller scale compared to vertebrates, they provide one of the best examples of a cause and link to population level effects. The phenomenon of imposex (where females develop male genitalia and male organs are superimposed over female organs) was found to be the result of exposure to tributyl tin (TBT), an antifouling paint for ships, in the American mud-snail (*Nassarius obsoletus)* in US harbours and female dogwelks (*Nucella lapillus*) in the UK (Matthiessen *et al.*, 1995; Santillo *et al.*, 2001). Approximately 150 species of gastropods affected by imposex have been found across the world (Vos *et al.,* 2000).

Very low concentrations of TBT induce imposex and the mechanism of induction, which is still controversial, has been the subject of different hypotheses including: aromatase inhibition; testosterone excretion-inhibition; functional disorder of the female cerebropleural ganglia and involvement of amidated tetrapeptide Ala-Pro-Gly-Trp-NH2 (see Nishikawa, 2005, and references therein). However, results from unpublished work (Horiguchi, T, Katsu, Y. Ohta, Y and Iguchi, T) suggest that neither inhibition of aromatase by TBT nor androgen action by TBT is the principle cause of imposex in gastropods, namely rock shell *Thais clavigera* (Iguchi, 2006). In addition, the concentration at which aromatase inhibition occurs (micromolar) is not low enough to explain imposex (nanomolar concentration). The low-dose effects are likely to be mediated by receptors (Kanayama *et al.,* 2005).

Kanayama *et al.* (2005) applied the CoA-BAP system (a novel rapid ligand *in vitro* screening method, see Kanayama *et al.,* 2003) to evaluate 16 human nuclear receptors and 40 suspected EDCs. TBT strongly activated the RXR and the peroxisome proliferator-activated receptor (PPAR) gamma in a mammalian reporter gene assay. The effect of TBT on RXR was as strong as that of its endogenous ligand 9-*cis* retinoic acid in inducing the transactivation function of RXR in mammalian culture cells. This study indicates that TBT may exert its toxic effects through the activation of the PPAR gamma/RXR signalling pathway in mammals. Could it potentially operate by a similar mechanism in invertebrates?

The RXR homologue was cloned from rock shell *T. clavigera* (Nishikawa *et al.,* 2004). The gastropod RXR showed great similarity (highest similarity in the DBD) to vertebrate RXRs (90% human, 88% fish and fruit fly and 87% chicken and frog). The highest similarity of the LBD was to the human RXR (84%). In light of this similarity, it was not unexpected when *Thai* RXR bound its natural ligand, 9-*cis* retinoic acid, and induced imposex in females. Bouton *et al*. (2005) isolated a functional RXR orthologue from the mollusc *Biomphalaria glabrata* that also bound 9-*cis* retinoic acid.

These results suggest that a functional homologue of RXR in gastropods plays an important role in the induction, differentiation, and growth of male genital organs in female gastropods. Gene manipulation studies are needed to determine the precise role of RXR in the development of imposex in gastropods.

4.6.2 Molecular approaches unveil steroid nuclear receptors in molluscs

Steroid hormone receptors were thought to be vertebrate specific, since they were absent from fully sequenced genomes of invertebrates. Furthermore, invertebrates appear to rely on non-steroidal hormones (and receptors) to regulate biological functions (de Fur, 2004). However, there is evidence that steroid hormone receptors play an endocrine role (Jobling *et al.,* 2004). Recently, the ramshorn snail *Marisa*

cornuarietis was discovered to be sensitive to oestrogenic substances such as bisphenol A (BPA), which causes superfeminisation at concentrations as low as 1 μg/L (Oehlmann *et al.*, 2000). Characteristics of superfeminisation include additional female organs, enlarged accessory sex glands, gross malformations of the pallial oviduct and a stimulation of egg and clutch production, resulting in increased female mortality. This leaves the molecular mechanisms of steroid action in invertebrates unresolved.

Thornton *et al*. (2003) isolated an ER orthologue from the sea hare mollusc, *Aplysia californica*. They used degenerate PCR and RACE to isolate an ER sequence from adult neural tissues and ovotestes using primers derived from vertebrate ERs. The protein sequence of the DBD of the receptor was similar to the vertebrate ER (88% and 85% to ERα and β respectively). The P Box within the DBD that mediates recognition of response elements by oestrogen and other steroid receptors (SRs) was identical to that of the human ERs only. The LBD was less conserved, but was most similar to the vertebrate ER. The AF-2 activation domain, which is a small activation region within the LBD that mediates ligand interaction with coactivators, was nearly identical to the human ERs but not to those of other SRs or oestrogen related receptors (ERRs).

To characterise the functionality of the *Aplysia* ER, the DBD and LBD were analysed separately using reporter assays, by expressing them in fusion constructs in a cell culture system. The DBD was fused with a constitutive domain (AD) and cotransfected with ERE-luciferase reporter CHO-K1 cells (hamster cells). The LBD was fused with Gal4-DBD and cotransfected with a luciferase reporter driven by an upstream activator sequence (UAS) which is the response element for Gal4-DBD. *Aplysia* ER-DBD activated luciferase expression approximately 10-fold (relative to controls) more than the human ERα-DBD fusion. The *Aplysia* ER-LBD activated transcription constitutively, that is without any added ligand, oestrogen or other hormone (characteristic of an orphan receptor), and increased approximately 35-fold relative to controls. This was in contrast to the human ERα-LBD, which was only activated in the presence of oestrogen.

To understand SR evolution and the existence of both ligand-dependent and independent ERs, Thornton *et al.* (2003) synthesised and functionally characterised the conserved functional domains of the ancestral SR from which all extant SRs evolved. The ancestral SR had oestrogen receptor-like functionality, specifically binding oestrogen and activating transcription only when oestrogens were present, suggesting the constitutive activity of the *Aplysia* ER was a novel feature. The authors note that the existence of such an ER in invertebrates suggests that SRs may be present in many other invertebrates such as echinoderms, annelids, platyhelminthes and other molluscs and not restricted to vertebrates, suggesting a much broader range of animal taxa could be subject to endocrine disruption.

An important question remains unanswered and that is, what is the functional role of *Aplysia* ER's constitutive activity? There is very little information on the endocrinology of the sea hare, and there is no evidence that oestrogen or other steroids are present or play a biological role in *Aplysia* (Keay *et al.,* 2006).

To address the potential role of ERs in oestrogen sensitive molluscs (where evidence suggests oestrogens are likely to be of endocrine importance), Keay *et al.* studied the molecular characteristics of the ER in the cephalopod *Octopus vulgaris,* which is distantly related to *Aplysia*. Using a combination of degenerate PCR, RACE, electrophoretic mobility shift assay, reporter gene assays and ligand binding studies, they isolated an ER orthologue which was shown to have similar characteristics to those observed in *Aplysia*. It was a strong constitutive transcriptional activator from

conanical EREs. It did not bind oestradiol and was unresponsive to oestrogens and other steroid hormones.

This study shows that the *Octopus* ER is not likely to be a mediator of observed effects in *Octopus*; it is the presence of oestradiol and progesterone in *Octopus* tissues which correlates with reproductive status and there is also evidence of specific binding of oestradiol in reproductive tissues (see Keay *et al.,* 2006, and references therein). The authors state that such studies provide only indirect evidence that oestradiol is a functional hormone; it could be an intermediary product or metabolite of some other active hormone, and the binding observed could be due to some other protein such as an enzyme involved in endocrine signalling. There are numerous alternative pathways of steroid action which trigger a rapid non-genomic response that do not require transcriptional activation via classical steroid receptors (see Section 2.8).

The characterisation and functional role of ER orthologues in *Marisa cornuarietis* is currently being carried out using the same approach as Keay *et al.* (2006), by a research group at Brunel University in the UK (Routledge *et al.,* 2006). They have recently isolated an ER-like and an oestrogenrelated receptor-like (ERR) from *Marisa* which are highly similar in terms of their sequence and structure to vertebrate ERs and ERRs. Using Q-PCR and *in situ* hybridisation, the authors detected very high levels of mRNA in reproductive tissues, including tissues noticeably affected by the EDC tested. Further functional analysis is revealing interesting similarities and contrasts to the known functions of their vertebrate orthologues.

From the results on steroid receptors in invertebrates to date, constitutive activation appears to be a widespread discovery. It is important that future research examines the functional outcome of the mechanisms. In addition, research on the mechanisms of EDCs in invertebrates should also focus on other nuclear receptors, distinct from the ER and AR such as the RXR.

4.6.3 Examples of genomic applications in other invertebrate species used in biomonitoring

To assess gaps in information on endocrine disruption in invertebrates, the Department for Environment, Food and Rural Affairs (Defra) established a programme of research to address this and Johnson *et al.* (2005) published a final report entitled *Endocrine disruption in aquatic and terrestrial invertebrates*. The study examined endocrine disruption in the aquatic freshwater shrimp *Gammarus pulex* and the terrestrial earthworm *Eisenia* using novel genomic techniques. These techniques have helped to expand our knowledge of the endocrinology of the species examined, and both short and long-term reproductive studies have provided much needed data on suspected EDCs (oestradiol, ethinyloestradiol, testosterone, bisphenol A, nonylphenol, fenoxycarb, propoxur) and their effects on development and reproduction.

To summarise, *G. pulex* was shown to be a useful indicator species for the assessment of known or suspected EDCs. However, the complex inter-relationship between moulting and reproductive processes made the identification of biomarkers for ED more complex. The authors proposed that the relationship between processes and effects would best be elucidated using a microarray approach, such as a custom array containing biomarker genes from both males and females (this is being pursued through a NERC Case Studentship). Despite this difficulty, initial insights into the genetic components of the *Gammarus* endocrine system have been gained, with over 500 novel gene sequences identified.

Conversely, *Eisenia sp.* may not represent the most appropriate terrestrial species for assessing the effects of EDCs, despite being an important test species for assessing

the effects of substances on terrestrial ecosystems. For example, Ricketts *et al.* (2004) validated the annetocin gene as a biomarker of reproductive fitness in *Eisenia*. Annetocin is a neuropeptide normally expressed within tissues containing reproductive organs, and is involved in osmoregulation and egg laying. The human homologue is under the expressional control of the oestrogen receptor. No statistically significant effects on reproduction were observed for the EDCs tested. The inaptness of this species as a test species for EDC effects may be related to its hermaphroditic nature, where it appears to have inherent homeostatic mechanisms to compensate for internal fluctuations in oestrogen and androgen concentrations following exposure to EDCs (Johnson *et al., 2005*). As a result, the authors suggest the use of an alternative species, the collembolan (springtail) *Folsomia candida* (full report can be obtained from http://www.defra.gov.uk/science/Project_Data/DocumentLibrary/CB01007/CB01007_3268_ABS.doc).

The sequencing of invertebrate genomes such as the terrestrial model organism *C. elegans* genome, has boosted the use of DNA microarray techniques to study the whole genome response of *C. elegans*. Research on toxic responsive genes is providing new and important bioindicators for detecting EDCs in the environment. The nematode worm is widely used for genetic studies as well as acting as a living biomonitor in ecotoxicology, because of its widespread distribution, sensitivity to stress and ease of laboratory manipulation. Ecotoxicological uses of the worm include the use of transgenic strains with reporter gene assays to assess the expression of the general stress response gene *hsp*-16 under different kinds of stress (Link *et al.,* 1999; David *et al.,* 2003). Reichert and Menzel (2005) used a *C. elegans* whole genome array to investigate the suitability of a microarray as a toxicological approach to study the effects of xenobiotics. Five xenobiotics tested included known and potential EDCs (atrazine, PAHs β-naphthoflavone and fluoranthene, the drug clofibrate and the potent endocrine disrupter diethylstilboestrol – exposures all in the mg/L range). Under the test conditions, 203 genes belonging to different families like the cytochromes P450, UDP-glucoronosyltransferases, glutathionine *S*-transferases, carboxylesterases, collagens and other were upregulated, with fluoranthene inducing the most genes. A decrease in expression was observed for 153 genes, with atrazine having the strongest effect.

Professor John Craft and colleagues at Glasgow Caledonian University have been investigating endocrine disruption in the common mussel (*Mytilus edulis*). They are cloning an oestrogen-like receptor in the mussel and have been constructing cDNA libraries for gender species genes and assessing their expression throughout a reproductive cycle. The group are seeking to develop microarray technology to use as an environmental monitoring tool, to assess the impacts of chemicals in the common mussel. In order to construct the array, genomic data is required. Where this information is not available, suppression subtractive hybridisation (SSH) can generate cDNA libraries representative of pollutant responsive genes (Brown *et al.,* 2006). This lack of genomic data for sentinels can hinder the development of novel biomarkers at the nucleic acid level. The group have previously shown that SSH is a highly useful approach in fish where no genomic data are available (Brown *et al.,* 2004, 2004b).

In the mussel, SSH and a macroarray were used to investigate pollutant responsive genes in the digestive tract after exposure to 1 mg/L of the polycyclic aromatic hydrocarbon, benzo[*a*]pyrene (BaP). PAHs are thought to act via binding to the AhR in vertebrates, inducing gene expression including CYP1A. However, although AhR analogues have been described in the mussel they do not bind dioxins, suggesting an alternative mechanism in invertebrates (Brown *et al.,* 2006 and references therein). Following exposure to BaP, 112 and 25 clones (genes) were differentially expressed as upregulated and downregulated respectively. Gene ontology of the differentially

expressed genes revealed that a number of cellular mechanisms were affected. Genes included general stress response genes, heat shock proteins, metallothionein, transporter proteins and defensin which were upregulated. Other genes putatively identified included those implicated in xenobiotic metabolism, oxidative stress and lysosomal function and/or involved in redox cellular control in mammalian species. Essentially, BaP metabolism appears to induce genes involved in either oxidative stress defence mechanisms, or redox control of signal transduction and cellular signalling pathways (Brown *et al.,* 2006). The SSH approach has provided some insights into the mechanisms of toxicity of BaP in a sentinel species, and identified potential biomarkers of exposure that require further development.

Other methods at the development stage for the mussel include PCR, metabolomic and proteomic approaches. A vitellogenin assay is being developed based on a Q-PCR method, as an alternative to the traditional organic-labile phosphate assay and the immunoassay usually employed to detect vitellogenin levels in invertebrates; preliminary results look promising but further work is required (Puinean and Rotchell, 2006). Dr Mark Viant at the University of Birmingham is using metabolomics to develop a rapid and cost-effective predictive biomarker model for the mussel. The aim of the study is to create a mathematical model that will be able to predict whether a mussel is healthy, diseased or exposed to a specific chemical based on a metabolomic fingerprint of mussel tissue. Such new approaches have the potential to reduce the limitations in traditional approaches, which only detect a relatively small number of responses and can be prone to high variability in measurements.

Using a proteomics approach, Apriaz *et al.* (2006) analysed the digestive gland peroxisomal enriched fraction in *Mytilus edulis* after exposure to sublethal concentrations of the EDCs diallyl phthalate (DAP), 2,2',4,4'-tetrabromodiphenyl ether (PBD-47) and bisphenol A. Differentially expressed proteins were separated using fluorescent differential gel electrophoresis (DIGE) and identified using mass spectrometry (matrix-assisted laser desorption ionisation – time of flight (MALDI-TOF MS) or electrospray ionisation mass spectrometry (ESI MS/MS)). Protein expression signatures (PESs) were obtained for each exposure, and the results demonstrated that the proteins constituting a unique PES could be used as novel biomarkers to distinguish between these three model pollutants. In addition, a PES common to the three independent exposures was generated that could be applied to field experiments.

The *Daphnia* genome consortium (http://daphnia.cgb.indiana.edu/) is an international network of investigators aiming to mount the cladoceran, *Daphnia,* as a model system for evolutionary/ecological genetic and genomic studies. Since *Daphnia* have been used for toxicological studies for decades (tests are cheap and provide useful information), a wealth of information has been generated on their behaviour, ecology, population genetics, reproduction and physiology. This information can be integrated with genomic data, revealing cause and effect relationships of chemicals, and is key to the successful use of genomics in ecotoxicology. The consortium aims to provide genetic and genomic resources to study genes that affect ecological and evolutionary success in the environment. This includes the genome database WFleaBase (http://wfleabase.org/), whose primary species are *D. pulex* and *D. magna*. The database contains searchable and extractable information from EST projects, genome survey sequences and full genome sequencing projects such at *D. pulex*.

cDNA libraries to study gene expression are now being constructed and characterised from *D. magna* (Watanabe *et al.,* 2005). cDNA microarrays are being used to assess the molecular impact of the pesticide propicanazole using a reproductive-related cDNA of *D. magna*. Dr Amanda Callaghan and Professor Richard Sibley and collaborators at the University of Reading are working with *D. magna* using a cDNA microarray, to assess changes in gene expression as a result of exposure to stressors such as

cadmium, lufenuron, pH, hardness, kerosene and ibuprofen. These gene responses are being linked to population changes of *D. magna* using a rapid, non-destructive and validated imaging system. Differential display polymerase chain reaction (ddPCR, see glossary) is another successful approach (like SSH) for screening and analysing the expression of novel genes. Diener *et al.* (2004) have optimised a ddPCR protocol that has the advantage of only requiring submicrogram quantities of total RNA, which is less than 10 *Daphnia* per test, and it uses a sensitive fluorescent tagging system. The technique has promised to advance knowledge on gene expression responses to exposure to toxicants, and could be applied to study the effects of EDCs.

5 Summary

Each of the genomic technologies has advantages and limitations. Despite the limitations, probably the greatest advantage of using these techniques is the ability to determine the mechanism of action of a toxicant. Understanding the mechanisms of action of EDCs is critical, as this is central to determining whether the chemical is an endocrine disrupter and causes an adverse response in the individual or population.

Understanding these complex gene activation patterns and how EDCs exert their pressure, either in isolation or as mixtures, was not possible before the emergence of genomic technologies, such as global gene expression profiling using gene arrays. Understanding the mechanisms of action of EDCs will be crucial for determining the risk of a particular exposure. Through systematic efforts to generate mechanistic information, diagnostic and predictive assessment of the risk of EDCs will be established in model species for ecological risk assessment (Iguchi et al., 2005).

If we are to understand the molecular basis of ED in organisms, it is essential that we understand the links between exposure, gene response to exposure and most importantly, the adverse effect induced by exposure (Iguchi et al., 2005). Iguchi et al. state that understanding the effects of EDCs on various species from invertebrates to mammals is greatly needed.

Genomic tools have been used to study the effects of EDCs on model organisms, which benefit from having a significant amount of genomic resource already available, as well as toxicological data. The tools have also been used on ecologically relevant or sentinel species. The main applications have been to assess transcript responses (transcriptomics) either as multi-gene arrays or single gene expression studies, in response to exposure to potential or known EDCs (especially in fish). These studies have enabled researchers to identify and functionally characterise genes involved in endocrine disruption, and work towards developing biomarkers of ED. They have also been used to clone steroid and non-steroid receptors in wildlife, to study the function of hormone receptors and gain insights into underlying mechanisms *in vivo*.

Proteomic and metabolomic approaches have been less forthcoming in the study of endocrine disrupting chemicals, although they are on the increase. While a number of tools are available for studying changes in gene transcripts, proteins and metabolites, the techniques are at different stages of development and as a result, their potential integration into a regulatory context will be realised at different points in time. The feature article by Ankley *et al.* (2006) considers the role of toxicogenomics in regulatory ecotoxicology, explores current limitations of the technology and its integration into regulation, and looks at potential ways of resolving some of the major limitations. In terms of applying genomic tools in ecotoxicological testing regimes, at present they are not in a state to replace traditional methods, but can nevertheless enhance the type of information derived from an experiment. Advances in biomarker discovery and the development of new endpoints have been rapid with the rise of genomics and molecular biology. Sensitive measurements of gene expression within organisms are now possible through the use of Q-PCR, and genomics has brought a closer understanding of the mechanistic links between exposure to EDCs and biological effect. Section 4 of this report has presented examples of where genomic tools have been applied in ED research on wildlife. However, there are some important issues to consider when using tools such as gene arrays, as explained below.

5.1 Issues to consider when using gene arrays in ecotoxicology

Genomic tools are increasingly being used to identify mechanisms or pathways of disruption after exposure of wildlife to EDCs. Despite the efforts being directed to this area of research, the datasets publicly available are still very limited, partly because of the technical difficulties and costs associated with performing microarray experiments. The costs involved in obtaining the arrays and equipment to analyse samples are considerable, and this limits the number of laboratories with the capacity to perform such experiments. UK research councils have recognised this and have invested in a limited number of recognised UK research laboratories, to establish the required infrastructure for the wider user community

The planning and experimental design of studies involving genomics requires knowledge and understanding of both the animal's biology and the genomic techniques to be employed, along with associated bioinformatics to analyse the genomic data.In studies employing genomics, experiments need to be designed based on the requirements of the toxicology (for example, a dose response may need to be generated) and the complexities of the statistics required to analysed the array data. These two elements are not always compatible, and experiments with arrays also need to take into account time. Gene expression is highly dynamic, with some genes responding to a particular stressor almost immediately (within hours of the initiation of exposure, for example with genes involved in the immune system), whereas other genes respond in a much slower fashion (within days) as has been shown by Moggs and colleagues in mouse uteri exposed to oestrogens (Moggs *et al.*, 2004). Often the phenotypic effects take days, weeks or even months to manifest. It is therefore important to sample animals at the right time, for the relevant endpoints (genes, cellular responses, tissue responses), to establish the links between them.

5.1.1 Variation in gene expression between individuals

Individual variation, especially when considering the effects of chemicals on wild species, is likely to drive the number of individuals required in the test to numbers incompatible with the cost of genomic technologies. To reduce cost, many studies limit the number of samples analysed using genomic technologies, by pooling samples. This approach, however, severely restricts the value of subsequent datasets, and indeed power of analyses for biological interpretation. In a recent study on fish by Santos *et al.* (2006), individual gonadal transcriptomes of female gonads at equivalent stages of reproductive development were highly variable (Santos *et al.*, 2007b).

A variety of factors can contribute to differences in gene expression among individuals, including differences in immune status, sex, genotype and age, but work on the Common mummichog fish (*Fundulus heteroclitus*) has shown that most inter-individual variation in metabolism can be accounted for by differences in metabolic gene expression (reviewed by Whitehead *et al.*, 2005). In order to study individual variation, Whitehead *et al.* (2005) examined the expression of a suite of 192 metabolic genes in the brain, heart and liver in three populations of *F. heteroclitus* using a highly replicated experimental design (Whitehead *et al.*, 2005). Nearly half of the metabolic genes (48 per cent) were differentially expressed among individuals within a population for any one tissue, with fold differences ranging from one- to five-fold and *p*-values ranging down to 10^{-7} (Whitehead *et al.*, 2005).

5.1.2 Normalisation

In order to extract biological information from data obtained using genomic techniques, the data requires normalisation. Gene responses need to be normalised against genes that are not affected by the treatment (so-called internal control or 'housekeeping' genes) in order to account for differences in starting material, hybridisation, and so on. It is crucial to choose the control genes carefully, making sure that these genes are not affected by the treatment. A recent study by Filby *et al.* investigated the usefulness of four functionally distinct and widely used internal control/housekeeping genes (18S rRNA; *ribosomal protein l8, rpl8*; *elongation factor-1*α, *ef1a*; *glucose-6-phosphate dehydrogenase, g6pd*) in the fathead minnow (Filby *et al.*, 2007). Fathead minnow were exposed to 35 ng/L E2 and expression of the control genes was measured in various tissue samples, including brain, pituitary, gill, liver, gonad, intestine and muscle using real-time PCR. All four genes varied in their expression between tissue types, and only 18S rRNA was consistent between fish at different stages of sexual development. Exposure to E2 consistently downregulated *g6pd* in both liver and gonad, and 18S rRNA in gonad. Furthermore, the effects of E2 on expression of oestrogen receptor, *esr1*, were overestimated when *g6pd* was used for the normalisation (Filby *et al.*, 2007). This study showed that prevalidation of control genes is critical for accurate assessments of the expression of target genes following exposure to environmental oestrogens.

Transcriptomic profiling using microarrays is particularly sensitive to the type of normalisation employed, as hundreds to thousands of genes with biologically diverse functions might be wrongly interpreted as being differentially expressed. When gene arrays are employed to quantify large numbers of randomly selected transcripts (in excess of 1,000) powerful mathematical treatments of the dataset can be used to normalise gene expression prior to biological analysis. These transformations are based on the assumption that exposure to chemicals that cause sublethal alterations in the physiology of the organisms is likely to impact a small proportion of genes, and therefore the overall gene expression is likely to remain constant between treatments. These unaffected genes are very useful and enable powerful normalisation of the dataset, allowing for the biological responses to stressors to be identified correctly.

It is fundamental to address the normalisation of gene expression profiles in detail for each dataset and each technique, in order to generate reliable biological conclusions and avoid technical bias of the effects reported.

5.1.3 Annotation

A major setback in the biological interpretation of gene expression datasets obtained during exposure studies, is the annotation of gene sequences available in public databases. Often, even when commercial microarrays are employed, annotations of the probes contained in the array are incomplete. Frequently, the identity of the gene is unknown or it is based on homology to a gene in other species; only a relatively small proportion of genes are firmly identified and functionally described. This problem is particularly evident in fish gene databases, even for the best characterised genomes like the Fugu and zebra fish genomes, where annotation of the gene probes is often less than 50 per cent. This results in datasets where the biological significance of the gene lists found to be affected by the toxicant is impossible to ascribe. A successful

method of analysing the significance of alterations in gene expression caused by chemical exposure is the use of gene ontology terms in a statistical manner (Currie *et al.*, 2005). To do this, however, the terms associated with each transcript must be known. This tool is likely to be increasingly used to determine the biological significance of observed effects and with the increased genomic resources available, it is likely to become a tool of choice to standardise the interpretation of microarray datasets across studies.

5.1.4 Phenotypic anchoring

In order to evaluate the mechanisms and biological implications of chemical exposure, it is important to relate the gene responses observed to other phenotypic endpoints such as morphological and physiological parameters. The establishment of relationships between gene expression signatures or fingerprints of exposure with other phenotypic measures of exposure is known as phenotypic anchoring. This method has been successfully employed to elucidate the effects of exposure to EE2 in the mouse uterus, helping to unravel the biological mechanisms leading to the effects observed (Moggs *et al.*, 2004). In fish, such studies have been less forthcoming, due to the difficulties of conducting studies, including all endpoints required. Recent studies using zebra fish, however, identified mechanisms of disruption of gonadal function following exposure to EE2 in males and females, and proposed novel molecular mechanisms leading to the decrease in egg production and sperm quality observed in the same individual fish (Santos *et al.*, 2007b). Other studies are underway using microarray technology to address the effects of single chemicals and environmental mixtures in the roach, stickleback, flounder, and so on. These studies may identify novel biomarkers for simple screening tests (such as dipstick tests) to safeguard wild populations.

It is likely that after exposure to EDCs and other stressors, individual (gene) responses will include both adaptive and toxic processes, adding a new degree of complexity to the interpretation of the gene expression profiles obtained. In order to separate the toxic responses (deleterious for the individual and potentially for the population) from the adaptive responses (which may not affect the health of the fish and population), more information/data is needed on phenotypic anchoring of different classes of EDCs. In the current literature, there are no examples of studies in fish where this issue has been addressed, but it is fundamental to the interpretation of gene expression datasets and their use in legislative protection of the environment.

5.1.5 Basic biology

A shortfall in ecotoxicogenomic studies leads to a lack of information on the normal physiology of test organisms prior to exposure. Many studies only include small sets of control samples to compare with exposed samples. However, the complexity of the gene responses measured and the limited number of samples analysed means it is difficult to determine if the control gene expression profiles are following the expected patterns, or if they are affected in some way by the test conditions. Many factors may result in bias of datasets including the age, gender, developmental and physiological status, time of day when the samples were collected, photoperiod, temperature, nutritional status and disease status. Even when control and exposed individuals are kept and sampled under similar conditions, these factors may lead to specific responses to the toxicant. In order to generate reliable toxicological gene expression

datasets, it is fundamental to define the *normal* gene expression profiles under a range of conditions for the test organism employed. This is especially important for samples collected from, for example, wild fish populations, where the history of the animal is not known. Such *control* datasets will improve the analyses of toxicological datasets and help scientists to determine if the alterations observed are caused by exposure to the chemical or by other extrinsic factors.

6 Conclusion

Recent developments in genomics, especially in the field of ecotoxicogenomics, promise a new level of understanding of the mechanisms of toxicity, along with the ability to detect the effects of exposure to EDCs at lower toxicant concentrations in wildlife populations. However, further research is needed to establish and validate the use of genomics for environmental monitoring, before these tools can be considered for regulatory application. Research is needed to:

- validate the tools being developed;
- develop datasets for various species and environments, documenting normal gene expression profiles and fingerprints of exposure;
- further develop resources for analysing the datasets generated, and maintain publicly available databases to support work in this area of research;
- develop scientific partnerships of experts in ecotoxicology and genomics, in order to fully make use of the tools that these technologies are able to provide.

Among the most promising emerging technologies, microarrays and real-time PCR are likely to be especially useful for the detection, monitoring and mechanistic understanding of the risks posed by environmental chemicals. Microarrays can help elucidate the mechanisms of action of individual chemicals and identify new biomarkers for environmental monitoring, but their complexity and cost restricts their use to large specialised laboratories. Once molecular biomarkers are identified, real-time PCR can be applied to large numbers of samples at a relatively low cost, making this technique particularly suited for environmental monitoring.

The genome sequencing efforts of the last decade have opened new avenues for biologists in all areas of research. The potential for discovery is theoretically unlimited, as phenotypic responses at the cellular level are mediated in most cases by measurable changes in gene expression. However, progress in the application of these resources to ecotoxicology will depend on progress in our understanding of the relationships between gene expression alterations and phenotypic changes as a result of chemical exposure, and this area requires further research.

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List of Abbreviations

11-KT	11-ketotestosterone
AhR	arylhydrocarbon receptor
ARNT	arylhydrocarbon receptor nuclear translocator protein
B[*a*]P	benzo[*a*]pyrene
BDE-47	2,2,4,4'-tetrabromodiphenyl ether
BPA	bisphenol A
CAFOs	concentrated animal feeding operations
CYP1A	cytochrome P450 enzyme
DBD	DNA binding domain
DDE	1,1-dichloro-2,2-bis(chlorophenyl) ethylene
DDT	2,2-bis(chlorophenyl)-1,1,1- trichloroethane
DES	diethylstilbestrol
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid (cDNA = complimentary DNA)
E2	17β-oestradiol
ED	endocrine disruption
EDC	endocrine disrupting chemicals
EE2	17α-ethinyloestradiol
ER	oestrogen receptor
ERE	oestrogen response element
EROD	ethoxyresorufin-*O*-deethylase
GSI	gonadosomatic index
LBD	ligand binding domain
mRNA	messenger ribonucleic acid
MT	17α-methyltestosterone
NMR	nuclear magnetic resonance
OCPs	organochloride pesticides
OECD	Organisation for Economic Cooperation and Development
PAHs	polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PCDD	polychlorinated dibenzo-p-dioxin
PCDF	polychlorinated dibenzofuran
PCR	polymerase chain reaction
p,p'-DDE	*p,p'*-Dichlorodiphenyldichloroethylene
PR	progesterone receptor

Q-PCR	quantitative polymerase chain reaction
RT-PCR	real-time polymerase chain reaction
RXR	retinoic acid receptor
STW	sewage treatment works
TBT	tributyltin
TCDD	total tetrachlorodibenzo-p-dioxin
TDCs	thyroid-disrupting chemicals
TR	thyroid receptor
TRB	trenbolone
VTG	vitellogenin

Glossary

Amplification: an increase in the number of copies of a specific DNA fragment.

Annetocin: A neuropeptide hormone with high homology to members of the vasopressin/oxytocin superfamily of neurohypophysial hormones. Evidence suggests that annetocin elicits stereotypical egg-laying behaviours in some invertebrates including ovulation and oviposition.

Apoptosis: The most common form of physiological (as opposed to pathological) cell death. Apoptosis is an active process requiring metabolic activity by the dying cell. Often called programmed cell death, although this is not strictly accurate.

Bioinformatics: the science of informatics as applied to biological research. Informatics is the management and analysis of data using advanced computing techniques. Bioinformatics is particularly important as an adjunct to genomics research, because of the large amount of complex data this research generates.

Biomarker: observable change (not necessarily pathological) in the function of an organism, related to a specific exposure or event.

Chromosome: The DNA in a cell is divided into structures called chromosomes.Chromosomes are large enough to be seen under a microscope. In humans, all cells other than germ cells usually contain 46 chromosomes: 22 pairs of autosomes and either a pair of X chromosomes (in females) or an X chromosome and a Y chromosome (in males). In each pair of chromosomes, one chromosome is inherited from an individual's father and one from his or her mother.

Clone: A term which is applied to genes, cells, or entire organisms which are derived from - and are genetically identical to - a single common ancestor gene, cell, or organism, respectively. Cloning of genes and cells to create many copies in the laboratory is a common procedure essential for biomedical research. Note that several processes which are commonly described as cell 'cloning' give rise to cells which are almost but not completely genetically identical to the ancestor cell. 'Cloning' of organisms from embryonic cells occurs naturally in nature (e.g. with the occurrence of identical twins).

Cloning: the process of producing a genetically identical copy (clone).

Cloning vector: DNA molecule originating from a virus, a plasmid, or the cell of a higher organism into which another DNA fragment of appropriate size can be integrated without loss of the vectors capacity for self-replication; vectors introduce foreign DNA into host cells, where it can be reproduced in large quantities. Examples are plasmids, cosmids, and yeast artificial chromosomes; vectors are often recombinant molecules containing DNA sequences from several sources.

Codon: a set of three nucleotide bases in a DNA or RNA sequence, which together code for a unique amino acid. For example, the set AUG (adenine, uracil, guanine) codes for the amino acid methionine.

Cofactor: a component such as a metal ion required by an enzyme to function.

Complementary DNA (cDNA): Viral reverse transcriptase can be used to synthesize DNA that is complementary to RNA (e.g. an isolated mRNA). The cDNA can be used, for example, as a probe to locate the gene or can be cloned in the double-stranded form.

Deletion: in the process of DNA replication, a deletion occurs if a nucleotide or series of nucleotides is not copied. Such deletions may be harmless, may result in disease, or may in rare cases be beneficial.

DNA (Deoxyribonucleic Acid): the molecule that encodes genetic information. DNA is a double-stranded helix held together by bonds between pairs of nucleotides.

DNA probe: a piece of single-stranded DNA, typically labelled so that it can be detected (for example, a radioactive or fluorescent label can be used), which can single out and bind with (and only with) another specific piece of DNA. DNA probes can be used to determine which sequences are present in a given length of DNA or which genes are present in a sample of DNA.

Electrophoresis: A method of separating large molecules (such as DNA fragments or proteins) from a mixture of similar molecules. An electric current is passed through a medium containing the mixture, and each kind of molecule travels through the medium at a different rate, depending on its electrical charge and size. Separation is based on these differences. Agarose and acrylamide gels are the media commonly used for electrophoresis of proteins and nucleic acids.

Endocrine Disrupting Chemical: An exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function.

Exogenous DNA: DNA which has been introduced into an organism but which originated outside that organism (e.g. material inserted into a cell by a virus).

Exon: exons are those portions of a gene which code for proteins.

Expressed sequence tag (EST): a short strand of DNA (approximately 200 base pairs long) which is part of a cDNA. Because an EST is usually unique to a particular cDNA, and because cDNAs correspond to a particular gene in the genome, ESTs can be used to help identify unknown genes and to map their position in the genome.

Full gene sequence: the complete order of bases in a gene. This order determines which protein a gene will produce.

Gene: a length of DNA which codes for a particular protein, or in certain cases a functional or structural RNA molecule.

Gene expression: The process by which a gene's coded information is converted into the structures present and operating in the cell. Expressed genes include those that are transcribed into mRNA and then translated into protein and those that are transcribed into RNA but not translated into protein (e.g., transfer and ribosomal RNAs).

Gene Families: Groups of closely related genes that make similar products.

Gene Library: A collection of cloned DNA fragments which, taken together, represent the entire genome of a specific organism. Such libraries or 'gene banks' are assembled to allow the isolation and study of individual genes. Gene libraries are produced by first breaking up or 'fractionating' an entire genome. This fractionation can be accomplished either by physical methods or by use of restriction enzymes. The genome fragments are then cloned (multiplied in number) and stored for later use.

Gene sequence: The order of nucleotide bases in a DNA molecule that constitute a gene.

Genetic Code: the set of codons in DNA or mRNA. Each codon is made up of three nucleotides which call for a unique amino acid. For example, the set AUG (adenine,

uracil, guanine) calls for the amino acid methionine. The sequence of codons along an mRNA molecule specifies the sequence of amino acids in a particular protein.

Genetic Mutation: a change in the nucleotide sequence of a DNA molecule. Genetic mutations are a kind of genetic polymorphism. The term 'mutation', as opposed to 'polymorphism', is generally used to refer to changes in DNA sequence which are not present in most individuals of a species and either have been associated with disease (or risk of disease) or have resulted from damage inflicted by external agents (such as viruses or radiation).

Genetic Polymorphism: a difference in DNA sequence among individuals, groups, or populations (e.g. a genetic polymorphism might give rise to blue eyes versus brown eyes, or straight hair versus curly hair). Genetic polymorphisms may be the result of chance processes, or may have been induced by external agents (such as viruses or radiation). If a difference in DNA sequence among individuals has been shown to be associated with disease, it will usually be called a genetic mutation. Changes in DNA sequence which have been confirmed to be caused by external agents are also generally called 'mutations' rather than 'polymorphisms'.

Genomic DNA: The basic chromosome set consisting of a species-specific number of linkage groups and the genes contained therein.

Genome: all the genetic material in the chromosomes of a particular organism; its size is generally given as its total number of base pairs.

Genomics: the study of genes and their function. Recent advances in genomics are bringingabout a revolution in our understanding of the molecular mechanisms of disease, including the complex interplay of genetic and environmental factors. Genomics is also stimulating the discovery of breakthrough healthcare products by revealing thousands of new biological targets for the development of drugs, and by giving scientists innovative ways to design new drugs, vaccines and DNA diagnostics. Genomics-based therapeutics include 'traditional' small chemical drugs, protein drugs, and potentially gene therapy.

Genotype: the particular genetic pattern seen in the DNA of an individual. 'Genotype' is usually used to refer to the particular pair of alleles that an individual possesses at a certain location in the genome. Compare this with phenotype.

Housekeeping genes: The genes which are expressed in all cells and which code for molecules that are necessary for basic maintenance and essential cellular functions.

Human Genome Project: an international research effort aimed at discovering the full sequence of bases in the human genome. Led in the United States by the National Institutes of Health and the Department of Energy.

Hybridisation: The process of joining two complementary strands of DNA or one each of DNA and RNA to form a double-stranded molecule.

In Situ Hybridisation (ISH): Use of a DNA or RNA probe to detect the presence of the complementary DNA sequence in cloned bacterial or cultured eukaryotic cells.

Intersex: Condition where both male and female features occur in the same gonad.

Library: a set of clones of DNA sequences from an organism's genome. A particular library might include, for example, clones of all of the DNA sequences expressed in a certain kind of cell, or in a certain organ of the body.

Ligand: A chemical (or molecule) that binds to a recptor. Ligands can be agonists or antagonists.

Macroarray: A low-density array of DNA molecules used for parallel hybridisation analysis (see microarray).

Marker: a sequence of bases at a unique physical location in the genome, which varies sufficiently between individuals that its pattern of inheritance can be tracked through families and/or it can be used to distinguish among cell types. A marker may or may not be part of a gene. Markers are essential for use in linkage studies and genetic maps to help scientists to narrow down the possible location of new genes, and to discover the associations between genetic mutations and disease.

Messenger RNA (mRNA): the DNA of a gene is transcribed (see transcription) into mRNA molecules, which then serve as a template for the synthesis of proteins.

Metabolome: constituent metabolites in a biological sample.

Metabolomics: techniques available to identify the presence and concentrations of metabolites in a biological sample.

Microarray (also known as gene or genome chip, DNA chip, or gene array): a component of a device for screening genomic or cDNA for mutations, polymorphisms or gene expression. The array is a small glass slide or other solid surface on which thousands of immobilised oligodeoxynucleotide probes have been synthesized or robotically deposited in a predetermined array, so that automated recording of fluorescence from each of the spots may score successful hybridisations. An array may be designed for the detection of all known genes of a species or selected specific sequences. The array may also consist of different antibodies or proteins.

Mutation: A change, deletion, or rearrangement in the DNA sequence that may lead to the synthesis of an altered inactive protein the loss of the ability to produce the protein. If a mutation occurs in a germ cell, then it is a heritable change in that it can be transmitted from generation to generation. Mutations may also be in somatic cells and are not heritable in the traditional sense of the word, but are transmitted to all daughter cells.

Nucleic Acid: one of the family of molecules which includes the DNA and RNA molecules. Nucleic acids were so named because they were originally discovered within the nucleus of cells, but they have since been found to exist outside the nucleus as well.

Nucleotide (= base): the 'building block' of nucleic acids, such as the DNA molecule. A nucleotide consists of one of four bases - adenine, guanine, cytosine, or thymine - attached to a phosphate-sugar group. In DNA the sugar group is deoxyribose, while in RNA (a DNArelated molecule which helps to translate genetic information into proteins), the sugar group is ribose, and the base uracil substitutes for thymine. Each group of three nucleotides in a gene is known as a codon. A nucleic acid is a long chain of nucleotides joined together, and therefore is sometimes referred to as a 'polynucleotide'.

Nucleus: the membrane bound structure containing a cell's central DNA found within all eukaryotic cells.

Oestradiol (= estradiol (USA); follicular hormone): A hormone synthesized mainly in the ovary, but also in the placenta, testis, and possibly adrenal cortex. A potent oestrogen.

Oestrogen (= estrogen (USA): A steroid sex hormone that regulates female reproductive processes and creates feminine secondary sexual characteristics. Some types of cancer depend on oestrogen for their growth, and modern-day increases in cancer rates are thought by many to be caused by the action of certain chlorinated

organic chemicals (such as pesticides and herbicides) that mimic the action of this hormone.

Oligonucleotide: A molecule made up of a small number of nucleotides, typically fewer than 25. These are frequently used as DNA synthesis primers.

Oxytocin: A peptide hormone from hypothalamus: transported to the posterior lobe of the pituitary. Induces smooth muscle contraction in uterus and mammary glands.

PCR: See polymerase chain reaction.

Phenotype: A set of observable physical characteristics of an individual organism. A single characteristic can be referred to as a 'trait', although a single trait is sometimes also called a phenotype. For example, blond hair could be called a trait or a phenotype, as could obesity. A phenotype can be the result of many factors, including an individual's genotype, environment and lifestyle, and the interactions among these factors. It is the observed manifestation of a genotype. The phenotype may be expressed physically, biochemically, or physiologically.

Polymerase chain reaction (PCR): The first practical system for *in vitro* amplification of DNA, and as such one of the most important recent developments in molecular biology. Two synthetic oligonucleotide primers, which are complementary to two regions of the target DNA (one for each strand) to be amplified, are added to the target DNA (that need not be pure), in the presence of excess deoxynucleotides and Taq polymerase, a heat-stable DNA polymerase. In a series (typically 30) of temperature cycles, the target DNA is repeatedly denatured (around 90°C), annealed to the primers (typically at 50-60°C) and a daughter strand extended from the primers (72°C). As the daughter strands themselves act as templates for subsequent cycles, DNA fragments matching both primers are amplified exponentially, rather than linearly. The original DNA need thus be neither pure nor abundant, and the PCR reaction has accordingly become widely used not only in research, but in clinical diagnostics and forensic science.

Probe: Single-stranded DNA or RNA molecules of specific base sequence, labelled either radioactively or immunologically, that are used to detect the complementary base sequence by hybridisation.

Promoter: A segment of DNA located at the 'front' end of a gene, which provides a site where the enzymes involved in the transcription process can bind on to a DNA molecule, and initiate transcription. Promoters are critically involved in the regulation of gene expression.

Proteome: Total protein complement expressed by a cell, tissue or organism.

Proteomics: Study of protein properties on a large scale to obtain a global, integrated view of cellular processes including expression levels, post translational modifications, interactions and location.

Real-time PCR: Also known as quantitative (real-time) PCR (Q-PCR). DNA is quantified after each round of amplification in ‘real-time’. When combined with reverse transcription, it is used to rapidly quantify mRNA facilitating the quantification of gene expression.

Recombinant DNA: DNA molecules that have been created by combining DNA from more than one source.

Restriction enzymes (restriction endonucleases): A class of bacterial enzymes that cut DNA at specific sites.

RNA (ribonucleic acid): A molecule similar to DNA, which helps in the process of decoding the genetic information carried by DNA.

Sequencing: Determining the order of nucleotides in a DNA or RNA molecule, or determining the order of amino acids in a protein.

Species: Groups of populations (which are groups of individuals living together that are separated from other such groups) which can potentially interbreed or are actually interbreeding, that can successfully produce viable, fertile offspring (without the help of human technology).

Toxicogenomics: A new scientific subdiscipline that combines the emerging technologies of genomics and bioinformatics to identify and characterise mechanisms of action of known and suspected toxicants. Currently, the premier toxicogenomic tools are the DNA microarray and the DNA chip, which are used for the simultaneous monitoring of expression levels of hundreds to thousands of genes.

Transcript: mRNA synthesised from a fragment of DNA.

Transcription: The process during which the information in a length of DNA is used to construct an mRNA molecule.

Transcriptomics: Techniques available to identify mRNA from actively transcribed genes.

Transcriptome: mRNA from actively transcribed genes.

Transcript profiling: (Also known as expression profiling) see transcriptomics.

Transformation: A process by which the genetic material carried by an individual cell is altered by incorporation of exogenous DNA into its genome.

Transgenic: An organism whose genome has been altered by the inclusion of foreign genetic material. This foreign genetic material may be derived from other individuals of the same species or from wholly different species. Genetic material may also be of an artificial nature. Foreign genetic information can be added to the organism during its early development and incorporated in cells of the entire organism. As an example, mice embryos have been given the gene for rat growth hormone, allowing mice to grow into large adults. Genetic information can also be added later in development to selected portions of the organism. As an example, experimental genetic therapy to treat cystic fibrosis involves selective addition of genes responsible for lung function and is administered directly to the lung tissue of children and adults. Transgenic organisms have been produced that provide enhanced agricultural and pharmaceutical products. Insect-resistant crops and cows that produce human hormones in their milk are just two examples.

Transgenic organism: An organism whose genome has been altered by the incorporation of foreign, or exogenous DNA.

Translation: The process during which the information in mRNA molecules is used to construct proteins.

Vector: [1] An organism which serves to transfer a disease-causing organism (pathogen) from one organism to another. [2] A mechanism whereby foreign gene(s) are moved into an organism and inserted into that organism's genome. Retroviruses such as HIV serve as vectors by inserting genetic information (DNA) into the genome of human cells. Bacteria can serve as vectors in plant populations.

Vitellogenin: A protein, precursor of several yolk proteins, especially phosvitin and lipovitellin in the eggs of various vertebrates, synthesized in the liver cells after oestrogen stimulation. Also found in invertebrates.

Xenobiotic(s): Substances not normally present in the reference organism.

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