EEA Technical report | No 2/2012

The impacts of endocrine disrupters on wildlife, people and their environments

The Weybridge+15 (1996–2011) report

ISSN 1725-2237

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Design: EEA
Layout: EEA/Pia Schmidt
Cover photo: © istockphoto/Martin Huber

Luxembourg: Publications Office of the European Union, 2012

ISBN 978-92-9213-307-8
ISSN 1725-2237
doi:10.2800/41462

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Acknowledgements

This report is an updated compilation of papers and discussions at the 'Weybridge+10' event in 2006, under the auspices of the Finnish Presidency of the European Union, co-organised by the European Commission's Directorate-General for Research (now Directorate-General for Research and Innovation), the Academy of Finland, and the European Environment Agency.

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Liability statement

Executive summary

Rates of endocrine diseases and disorders, such as some reproductive and developmental harm in human populations, have changed in line with the growth of the chemical industry, leading to concerns that these factors may be linked. For example, the current status of semen quality in the few European countries where studies have been systematically conducted, is very poor: fertility in approximately 40 % of men is impaired. There is also evidence of reproductive and developmental harm linked to impairments in endocrine function in a number of wildlife species, particularly in environments that are contaminated by cocktails of chemicals that are in everyday use. Based on the human and wildlife evidence, many scientists are concerned about chemical pollutants being able to interfere with the normal functioning of hormones, so-called endocrine-disrupting chemicals (EDCs), that could play a causative role in these diseases and disorders. If this holds true, then these 'early warnings' signal a failure in environmental protection that should be addressed.

In the 1996 Weybridge meeting on EDCs ('European Workshop on the Impact of Endocrine Disrupters on Human Health and Wildlife', European Environmental Agency (EEA)/Directorate-General for Research, 1996), the problem of endocrine disrupters was first comprehensively discussed by both European and United States regulatory authorities. Since then, substantial European Union (EU) funds (i.e. over EUR 150 million spent until 2011 have been allocated to research into endocrine disrupters and their effects, and the World Health Organization (WHO) and the Organisation for Economic Co-operation and Development (OECD) have addressed the problem in many ways. At the Weybridge meeting in 1996, much focus was placed on oestrogenic compounds, and especially on receptor-mediated effects. Scientific progress over the last decade or so has expanded the scope considerably: it includes EDCs that affect other hormone systems, e.g. the thyroid; EDCs with new modes of action, e.g. inhibitors of endogenous hormone production or metabolism; and target tissues for EDCs other than those in the reproductive system, such as the brain and cardiovascular system.

The Weybridge+10 workshop (Academy of Finland, European Commission's Directorate-General for Research and EEA, 2006) aimed to evaluate the impacts of this extensive research and to determine future goals in the areas of human and wildlife health effects, mechanisms of biological actions and models, exposures, risks, and policy options. Invited participants came from 18 European countries plus Israel, Japan and the USA, with representation from 41 Europe-funded projects related to EDCs.

The detailed reviews of current knowledge show clearly that human male reproductive problems are increasing in many countries, and that environmental factors which disrupt the normal development of the foetal reproductive system are crucial in the pathogenesis of these diseases and disorders. Moreover, laboratory studies show that the reproductive systems of a broad range of vertebrate species (e.g. polar bears and fish) and some invertebrate species (e.g. snails, oysters and insects) are susceptible to EDCs, and that foetal/early exposure of animal models to these chemicals can reproduce the pathogenesis seen in some populations. In wildlife, particularly some fish species, the evidence linking exposure to chemicals with reproductive disorders and dysfunction is strong; in humans and some other species, research is still sparse, largely due to the length, cost and methodological difficulties of such studies. However, there are now several ongoing prospective studies that measure exposure to EDCs during foetal life and its' possible connection with reproductive diseases and developmental disorders during childhood and adult life.

The realisation that chemicals can disrupt the normal development and function of the male reproductive system has led to much more investigation of the possible effects of EDCs on other endocrine diseases and disorders: exposure to oestrogen or to oestrogenic EDCs is an accepted risk factor for breast cancer, endometriosis, fibroids and polycystic ovarian syndrome (PCOS) in women. There are now limited data to support a role of xenoestrogens in the disease processes behind some of these disorders. Moreover, studies associating precocious puberty in girls with high levels of persistent dichlorodiphenyltrichloroethane (DDT) derivative *p,p'*-DDE (immigrant children) and polybrominated biphenyl (PBB) also exist. The increased incidence of diseases and disorders of the thyroid, immune, digestive, cardiovascular, and metabolic systems, together with laboratory studies

suggesting that EDCs could affect these systems, have led to further investigation of these areas. There are also limited numbers of epidemiological studies linking neurodevelopmental disorders in particular as well as thyroid disease with exposure to endocrine disrupters (ED). Some studies suggest that even minor changes in thyroid homeostasis in pregnant women may give rise to a reduction in cognitive function in children. Extensive studies with polychlorinated biphenyls (PCBs) have all reported negative associations between prenatal PCB exposure and measures of cognitive functioning in infancy or childhood.

In the last 10 years, many new computational predictors and both *in vitro* and *in vivo* assays for EDCs have been developed that greatly enhance the ability to study mechanisms of action and to screen large numbers of new and existing chemicals for hormone activity, so as to ensure their safety. Risk assessment and regulatory frameworks for dealing with EDCs have been developed. We have realised that there are characteristics typical of EDCs that make risk assessment processes difficult, such as critical time windows for exposure, the long latency between exposure and effect, and the realisation that every similarly acting EDC in a combination contributes to the overall mixture effect. In particular, the latter challenges the traditional risk assessment paradigm of a threshold dose below which a chemical fails to produce effects.

Human epidemiological studies on the association between exposure to EDCs and thyroid cancer, obesity, diabetes and/or metabolic syndrome are needed, as the rates of all of these diseases and disorders are steeply increasing. More studies in wildlife species are also needed, especially in view of the general decline in biodiversity and the possibility of ED involvement in this decline, but also because wildlife is a valuable sentinel of human health, and is much more easily and cheaply studied. Studies to investigate the impacts of chemical ED on invertebrate populations are a much neglected area of study, despite the fact that the first ever example of ED caused a catastrophic decline in — and even local extinction of — many mollusc populations in various parts of the world. The biggest knowledge gaps and priorities for research are found in the areas of exposure measurement and understanding, and in the development of an appropriate epidemiological framework, taking into account the possibility of multiple causality, latency and low-dose effects. Preclinical markers of disease are needed, in particular biomarkers of thyroid disruption that are independent of serum hormone levels. Environmental chemicals often interfere with thyroid hormone signalling without causing measureable changes in serum thyroid hormones, and there are currently no biomarkers for this. Moreover, the considerable controversy that still exists over the existence, relevance and reproducibility of non-monotonic U or inverted U-shaped dose–response curves needs resolution. Although considerable progress has been made in identifying EDCs, our knowledge about the nature of EDCs relevant to human and wildlife exposure scenarios is still far from complete.

Several key conclusions, challenges and recommendations have been drawn from the research over the last 14 years, not least of which is the conclusion that that chemically induced ED likely affects human and wildlife endocrine health the world over. A much better understanding of the role of exposure to environmental contaminants in the prevalence and risk of endocrine disease in both humans and wildlife is needed if we are to protect ourselves and wildlife from any harm caused by these chemicals.

1 General introduction

1.1 Endocrine disruption and the European Union

Genital malformations in baby boys have been increasing in many European countries, and the number of people diagnosed with breast, testis and prostate cancers continues to rise. Recent data indicate that in parts of Europe, sperm quality is approaching crisis levels that may impair fertility. At the same time, there is a secular trend towards earlier onset of puberty in young girls, and a steep increase in the paediatric rates of endocrine nutritional and metabolic disorders such as type II diabetes and obesity. Thyroid cancer rates have increased by between 5.3 % (Switzerland) and 155.6 % (France), particularly in females, children and young adults. Similarly, congenital hypothyroidism and neurodevelopmental disorders such as autism and attention deficit disorder are much more prevalent than they were 20 years ago.

The trends in the incidences of these endocrine diseases have changed in a manner concomitant with the rapid expansion in growth of the chemical industry, leading to growing speculation that these factors may be linked. There is also compelling evidence of compromised development, growth and reproduction in a number of wildlife species, with reports of alterations and abnormalities in sexual development, impaired thyroid function, and thyroid abnormalities, particularly in environments contaminated by cocktails of chemicals in everyday use.

The concern is that chemicals able to interfere with the normal functioning of hormones, i.e. EDCs, may play a role in these conditions. Such chemicals can be found in food, household products and cosmetics. The apparent parallels between effects reported in humans and wildlife populations are not surprising, given the overlap between their environments and their food chains.

To investigate the potential harmful effects of EDCs, the EU has embarked on extensive research efforts and to date has launched scientific projects worth more than EUR 150 million. The intention has been to provide the EU with the information it needs to ensure the safety of chemicals in use and to be used in the future. A lot of this research has now been completed and new findings concerning the effects of chemicals, especially when present as cocktails, have emerged. Progress has also been made in pinpointing human life stages particularly vulnerable to ED, and new data about endocrine health in both humans and wildlife have come to light.

1.2 Workshop overview and objectives

The Weybridge+10 workshop was named after the first workshop of its kind, the 'European Workshop on the Impact of Endocrine Disrupters on Human Health and Wildlife', which was held 10 years earlier in Weybridge, United Kingdom. Following the 1996 Weybridge meeting, a substantial amount of funds from the EU was allocated to research in this area and it became timely to evaluate the impacts of this extensive research and determine future research goals. The Weybridge+10 workshop aimed to produce a consensus document on the impacts of EDCs, the research results reached, hypotheses clarified, development in the area and future goals. Modelled on the 1996 Weybridge meeting and report structure, four working groups were set up to address the following: a) human health effects, b) wildlife effects, c) mechanisms and models, and d) exposure, risk and policy.

A preliminary meeting was held in Kos, Greece from 9 May to 11 May 2006 and as a starting point, recommendations missing from the 1996 Weybridge report were discussed (Table 1.1) and the report structure and task allocation clarified.

As part of the Finnish Council (of the European Union) Presidency, an expert meeting was organised and hosted by the Academy of Finland, with cooperation from the EU. Funding was provided by the Finnish Ministry of Social Affairs and Health, the EEA, the German Umweltbundesamt (UBA — the Federal Environment Agency), and the European Commission's Directorate-General for Research. Invited participants hailed from 18 European countries plus Israel, Japan and the USA, with representation from 41 European funded projects relating to ED, the European Commission, the OECD, the United States Environmental Protection Agency (US EPA) and the US National Institute of Environmental Health Sciences (NIEHS). Attendees included selected experts, scientists and

Table 1.1 Missing recommendations from the 1996 Weybridge report

Area	Recommendations not covered in 1996 Weybridge report
Human epidemiology	Female endpoints (endometriosis, PCOS)
	Immune system, thyroid
	Metabolic syndrome (obesity)
	Puberty (early onset in girls/late onset in boys)
	Neurodevelopmental disorders with EDCs other than PCBs
	Investigation of whether cancer is linked to mothers with boys
	Prostate cancers
Wildlife	Classes other than androgens and oestrogens require investigation (thyroid effects in laboratory tests (OECD) but not in the field studies)
	Community effects rather than population effects Identification
Mechanisms and models	Biomarkers for ovarian function
	Thyroid function in relation to neurotoxins
	The need for animal models other than those listed above, e.g. for hormone production, reproductive tracts other than the testis, ovary and prostate. How hormones affect the brain: gender assignment/intersex issue
	Epigenetics (relation to ED is uncertain) and transgenerational effects
	Obesity and puberty (considered together and independently)
Exposure	The need for new exposure assessment strategies, particularly to take account of mixed exposures
	Timing and duration (e.g. low level, long term) of exposure
	Methods with which to measure doses at target *in vivo*
	Call for establishment of national specimen banks
Methodology	Low-dose issue
	Hormone-independent activation of steroid receptors
	Interaction between substances, mixture effects, crosstalk of receptors
	Cell membrane–mediated effects
	Better integration between ED field and basic cancer research
	Development and validation of test guidelines (takes long time; e.g. Hershberger test took 10 years). Require optimisation of entire procedure by EU
	Effects on those beta cells that produce insulin, which could be linked to obesity
	Focus on active chemicals and their effects rather than on mechanisms of action

policymakers from the EU Member States, and international experts.

This report was distributed to all participants attending the Weybridge+10 workshop, the 'Impacts of Endocrine Disrupters', held from 8 November to 10 November 2006 in Helsinki, Finland. From each working group, a summary and the background review of research undertaken during the past decade was given. These background reviews were then subsequently updated to coincide with the 2012 publication of this report. All parts of this document have been peer-reviewed by experts in each of the areas discussed.

The objectives of the workshop were to:

- provide a forum for informal international discussion on research and testing of EDCs;
- summarise research progress made in the previous decade;
- assess the implications for risk assessment and policy;
- identify knowledge gaps;
- define future research priorities.

2 Review summaries

2.1 Human health effects

2.1.1 Introduction

From a human health perspective, the emphasis at the time of the Weybridge workshop in 1996 was on the observed temporal and geographical trends in male reproductive health. These included the well-documented trend of increasing incidence of testicular cancer, the indications of declining sperm count, and the suspicion that the incidence of undescended testes and hypospadias was increasing in the Western part of the world. It was also recognised that differing male reproductive disorders might be biologically associated: for example, testicular cancer is more common in patients with cryptorchidism, and vice versa. The documented increase in the incidence of breast cancer was also noted. It was stressed, however, that there was no evidence linking the observed changes in human reproductive health to exposure to EDCs, and that the observed changes may be caused by factors unrelated to EDCs. However, as the changes have occurred over a few generations, it is likely that the changing environment (including changes in lifestyle) may be responsible. Over a decade later, more progress has been made and more data now exist to support the ED hypothesis. These are summarised below.

2.1.2 Progress summary

Male relevant endpoints

Male reproductive health problems often occur simultaneously or sequentially, and increasing evidence now points to their origin in foetal development and early postnatal development. Testicular dysgenesis syndrome (TDS) encompasses poor semen quality, hypospadias, cryptorchidism and testicular cancer. These adverse conditions in male reproductive health often have a common aetiology relating to specific errors during the development of foetal testes. During the past 10 years, increasing evidence has accumulated showing adverse trends and geographical differences in TDS-related male reproductive health problems. These trends point very strongly towards an environmental cause working over a background of varying genetic susceptibility. Some evidence exists to implicate EDCs as causative factors in the development of this disease in animal models as well as in epidemiological studies.

Neonatal oestrogen exposure can permanently alter the development of the prostate gland in rodent models, and it is associated with increased incidence of hyperplasia, dysplasia and adenocarcinoma with ageing. Small doses of EDCs with oestrogen-like activity delivered to rodents *in utero* have been shown to result in prostate enlargement and/or inflammation, which then predisposes the prostate to neoplastic development. This notwithstanding, reliable human data linking foetal exposure to EDCs with prostate cancer are still lacking due to the difficulties involved in collecting this type of data. Since the first Weybridge meeting, however, an increased number of epidemiological studies report that serum concentrations of pesticides or phytoestrogens are associated with increased incidences of prostate cancer, although there seem to be geographical differences — studies in some countries indicate no such regional association. High intake of tomato carotenoids, mainly lycopene, seems to protect against prostate cancer. Lycopene has been ascribed multiple cellular actions including interaction with nuclear receptors, but its mechanism of action in the prostate remains undefined.

Female relevant endpoints

Breast cancer is the most common cancer in women, with incidence rates increasing in almost all industrialised countries. Genetic factors may account for up to 10 % of breast cancer cases in developed countries, but the majority of breast cancer cases are considered to be the consequence of lifestyle and environmental exposures. It is now well established that the cumulative dose of endogenous oestrogen that reaches a woman's breast tissue during her lifetime is a strong determinant of breast cancer risk. Exogenous oestrogen exposure is also a risk factor, as demonstrated by the increase in breast cancer attributable to hormone replacement therapy. These findings have heightened concerns about the role of environmental pollutants with oestrogenic activity.

A decade ago, epidemiological studies examining the risks associated with individual pollutants such as DDT and DDE were inconclusive. However, in

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contrast to a decade ago, there are now indications that increased breast cancer risk is associated with the body burden of all oestrogenic chemicals, excluding the natural hormones. There is growing evidence that the breast tissue is particularly vulnerable during development in the womb and puberty. Taken together, these findings all build a case for pursuing epidemiological studies of environmental pollution and breast cancer, but any such studies must take account of critical windows of vulnerability and of cumulative exposures to multiple EDCs. Ovarian cancer should also be focused on, as it shares many of the risk factors with breast cancer.

Compared with a decade ago, some progress has been made with breast cancer epidemiological studies, and several studies now report significant positive associations with blood levels of organochlorine (OC) pesticides and subsequent breast cancer incidence in women followed up for more than a decade after taking the initial blood sample. As with prostate cancer and TDS, if breast cancer is a disease of foetal origin caused by multiple similarly acting chemicals, the presumed large time lag between the (first) adverse exposure event and the manifestation of the disease, and the selection of the appropriate mix and number of chemicals, are potentially enormous challenges for the investigation of exposure–outcome relationships.

Polycystic ovarian syndrome (PCOS) is another major endocrine female disorder. Although the aetiology of PCOS remains unclear, it seems to involve hypersecretion of androgens during foetal development, thereby disrupting the hypothalamic-pituitary axis; this results in excess luteinising hormone (LH) secretion. Animal studies show that exposure of the foetus to excess androgens in the maternal circulation results in many of the features of PCOS as the animal reaches adolescence, indicating a possible role of exogenous factors leading to excess androgen action during the development/imprinting of the hypothalamic-pituitary-ovarian axis. However, human studies on the associations between exposure to EDCs and development of PCOS are lacking.

Endometriosis is an oestrogen-dependent disorder that can be modified by oestrogens. Some human studies have shown an association between exposure to PCBs and dioxins and endometriosis, but others, including a follow-up study from the 1976-Seveso accident investigating women exposed to high levels of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), have failed to show a statistically significant association. Hormonally active oestrogen-like substances can affect endometriosis, and there is a growing amount of evidence to support the part played by oestrogenic EDCs and phthalates in the pathogenesis of this disorder. Moreover, the benign proliferation of uterine smooth muscle cells causing uterine myomatosis and leiomyomas (fibroids) is also highly responsive to oestrogens, and it is tempting to suggest that oestrogenic compounds could also play a role in the pathogenesis of fibroids. Indeed, there are now limited data to support a role of xenoestrogens in the disease process behind this disorder.

Puberty

Observations associating exposure to EDCs with the timing and tempo of puberty has primarily been concerned with precocious puberty in girls who are much more prone to precocity than are boys. In most instances, the main route of exposure resulting in premature puberty has been with oestrogenic contamination of meat- or fish-based food resulting in peripheral puberty. Prevalence of central precocious puberty is exceptionally high for those children who have immigrated to Europe. Though the reason for this observation has not been identified, environmental factors are likely to play an important role. Exposure–outcome associations between early-onset puberty and high levels of persistent DDT derivative *p,p'*-DDE (in immigrant children) and polybrominated biphenyl (PBB) have been observed for girls. For boys, exposure–outcome relationships have remained less clear.

Possible effects on non-reproductive organs

Most research on endocrine disrupters to date has focused on the reproductive system, but the effects of these chemicals on other body systems are now under investigation, due to the increased incidence of diseases and disorders of these systems. The immune system, the digestive system, the cardiovascular system, the central nervous

system, metabolism, and fat (adipose) tissue are all targets of endocrine-disrupting compounds. Of these, the effects of environmental chemicals on thyroid function are the best investigated, due to the essential role played by thyroid hormone in brain development and the increased rates of neurodevelopmental disorders in children.

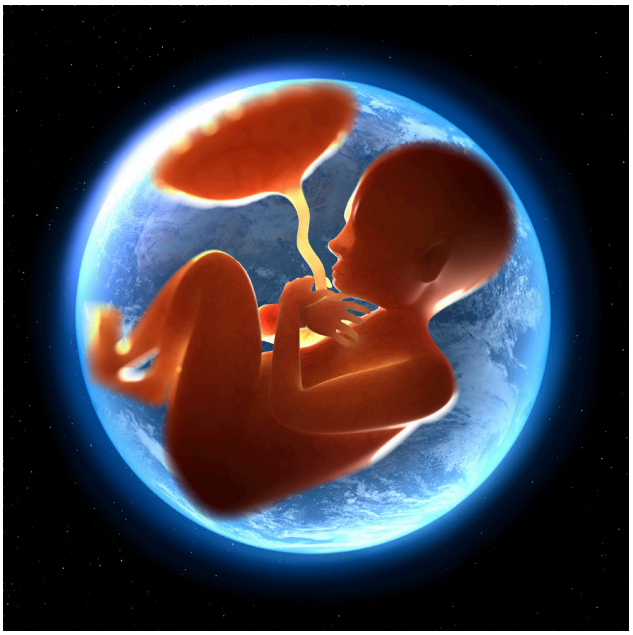
Many studies show associations between decreases in thyroid hormones and exposure to PCBs and dioxins. The foetal and newborn periods seem to be especially vulnerable developmental periods during which even small changes in thyroid hormone levels may have irreversible and detrimental effects on neurodevelopment. Except for the PCBs, however, there is currently not a convincing epidemiological case for exposures to EDCs playing a role in neurodevelopmental disorders or in the development of thyroid cancer, although its incidence is steeply increasing. Indeed, only five chemicals have been shown to be toxic to human neurodevelopment, despite the fact that there is experimental animal evidence for neurotoxicity of around 1 000 chemicals. It is highly likely that a mechanism for developmental neurotoxicity will involve thyroid disruption, and the biological plausibility of there being a role for endocrine disrupters to play in this seems high.

Thyroid hormone also plays a significant role in growth and metabolism, and the adrenal gland likewise in glucose and lipid metabolism. Other important endocrine organs are the pancreas and the adipose tissue. The significant and rapid increases in obesity, types I and II diabetes and metabolic syndrome have raised concerns; the increase is believed to be partially due to environmental factors. EDCs can influence lipogenesis, lipolysis, adipogenesis, leptin secretion or hormone synthesis by the liver through interaction with the glucocorticoid, sex steroid receptor or the peroxisome proliferator-activated receptor-gamma (PPARγ) (the major regulator of adipogenesis) systems. Excess oestrogen or oestrogenic compounds such as bisphenol A (BPA), for example, can produce an excess of insulin signalling and insulin secretion, overstimulating beta cells, and provoking increased insulin resistance and beta-cell exhaustion characteristic of type II diabetes. Taken together, animal studies clearly show a developmental origin for metabolic disorders, and proposed mechanisms of chemically induced metabolic disruption exist. This research notwithstanding, few human epidemiological studies on the association between exposure to EDCs and obesity, diabetes and/or metabolic syndrome have been carried out.

The adrenal cortex is under the control of the hypothalamic-pituitary axis, linking putative effects of EDCs in the brain to changes in glucocorticoid secretion and action. There is also an important interaction between the adrenal and the immune system: glucocorticoids are potent immune suppressors. Indeed, it has been proposed that EDC exposure during the development of the autoimmune system could cause type I diabetes in children.

There are no reports dealing with effects of EDCs on the adrenal medulla, but many studies indicate unwanted actions on the hypothalamic-pituitary-adrenal (HPA) axis. There is a great deal of literature on the effects of EDCs and other xenobiotics on adrenal and glucocorticoid receptor (GR) function in experimental animals; many different factors interfere with glucocorticoid homeostasis. DDT metabolites are well-known inhibitors of adrenal function, acting by direct cytotoxicity to adrenocortical cells. Emerging data in experimental animals indicate potent modulatory action of many EDCs on global steroidogenesis, including effects on adrenocortical cells.

With the exception of the glucocorticoids (e.g. insulin), however, immune cells are non-typical targets of traditional endocrine hormones, and the concept of ED is not well defined for this biological system. Numerous substances affect leucocytes, and it is difficult to distinguish toxic and immune activating/suppressive effects from those that may count as ED. Further, there are several reports demonstrating effects of EDCs on



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cytokine production in various systems. EDCs have been found to exert immune-modulating actions in both humans and experimental animals, and diethylstilbestrol (DES), polychlorinated dibenzo-p-dioxins (PCDDs), PCBs, dibenzofurans (PCDFs) and other substances have been shown to contribute. The observed effects are mostly weak and the mechanisms are most often obscure.

One common feature for several of the discussed possible human endpoints for ED is that although they may not manifest until adult life, they seem to be linked to events/exposures taking place during foetal or neonatal development.

2.1.3 Knowledge gaps and research priorities

Gaps

- Little exposure measurement during critical periods of sex differentiation, e.g. during first trimester of pregnancy.
- No epidemiological framework taking into account multiple causality, latency and low doses.
- No human biomonitoring for currently used pesticides, herbicides, polar xenoestrogens and phenolics shown to be endocrine disrupters in rodent assays.
- Preclinical markers for breast cancer and targeted research taking into account origins of breast cancer early in life and exposures to a multitude of chemicals.
- No epidemiological studies on fibroids and endometriosis.
- No biomarkers of thyroid disruption that are independent of serum hormone levels.
- No epidemiological studies of thyroid disruption linked to neurobehaviour, thyroid cancer, paediatric brain cancer, and metabolic and heart disease.
- No epidemiological studies of metabolic syndrome, obesity, and diabetes linked to EDCs.

Research priorities

- Link foetal/neonatal exposures to adult health outcomes by follow-up studies of available cohorts, taking account of recent advances in the understanding of EDC mixture effects.
- Identify early markers of adult reproduction (e.g. anogenital distance in baby boys related to sperm quality in adult men) in order to address long latencies of effects following exposures at critical time points during foetal and neonatal development.
- Conduct long-term surveillance of trends in semen quality and other endocrine diseases.
- Explore the link between semen quality and fertility/infertility rates in different populations, exploiting differences in regional prevalence to pinpoint contributing risk factors.
- Explore links between adverse different reproductive outcomes, e.g. testicular cancer, genital abnormalities and semen quality.
- Investigate the contribution of cumulative exposure to EDCs during critical windows of vulnerability to breast cancer risks.
- Develop new methods for the detection of EDCs in prepubertal children.
- Identify biomarkers of thyroid disruption that are independent of serum hormone levels.
- Perform epidemiological studies of thyroid disruption in development and adulthood linked to neurobehaviour, cancer (especially paediatric brain cancer), and metabolic and heart disease.
- Study how EDCs are linked to signalling pathways operating in obesity and regulation of appetite.
- Develop EDC biomonitoring strategies for human exposure and risk assessment, taking account of toxicokinetics and resultant levels in tissues and body fluids.
- Carry out studies on the link between individual foetal exposures and clinical outcomes.

2.1.4 Implications for risk assessment and regulatory action

Although proof of a link between human exposure to EDCs and disease outcomes is not available (and may never be), research conducted over the last 10 years has helped to substantiate the biological plausibility of such associations and to support the ED hypothesis: that exposure to

endocrine-disrupting contaminants is one of a number of multicausal factors involved in the manifestation of endocrine disease. Advances in our understanding of critical periods of vulnerability and of the cumulative effects of multiple EDCs acting in concert add to existing concerns about the health impacts of EDCs. The improved knowledge shows that the possible impacts of EDCs on human health cannot be dismissed and should be taken into account in risk assessment and regulation.

2.2 Wildlife effects

2.2.1 Introduction

At the time of the Weybridge meeting in 1996, with few exceptions, little was known about ED in wildlife. That situation has changed markedly in the last 10 years. Laboratory studies have shown that a broad range of species from vertebrate and invertebrate taxa are susceptible to ED chemicals; for many species, strong evidence exists to indicate that ED is a widespread phenomenon in wildlife populations.

In contrast with humans, in wildlife species, it is clear that there exist examples of male and female reproductive dysgenesis and of thyroid hormone disruption in some wildlife classes that can be linked, quite convincingly, to EDC exposure (although causation is difficult to prove). The symptoms reported appear to mirror those observed in the human population, indicating that the human and wildlife evidence should be considered in parallel when assessing whether EDCs contribute to the aetiology of endocrine diseases and disorders, and the risk posed.

2.2.2 Progress summary

The issue of ED effects in wild mammals has received relatively little attention. Predators have been studied in most detail because they are expected to have the highest contaminant burden as a consequence of biomagnification. Although this assumption has been confirmed, there is little evidence for ED as a consequence of this contaminant burden. This uncertainty arises in part because of the constraints placed upon such studies: the species of interest are often endangered and thus not available in sufficient numbers to provide adequate data to investigate causality. Observations of domestic animals inadvertently exposed to sewage-contaminated feed and studies on rodents in semi-natural enclosures provide indications that in principle, EDCs can cause population-level effects in wild mammals, and that novel approaches may be required to investigate this.

An extensive number of studies have continued to demonstrate clearly that ED in wild freshwater and marine fish is widespread. In most cases, observations have been associated with exposure to effluent from sewage treatment works (STWs), where steroidal oestrogens play a major role in causing ED. However, at specific locations, xenoestrogens can contribute significantly to the observed effects. EDCs can even cause population-level effects in some locales, impacting on reproductive output of these populations of fish. Most studies have focused on oestrogenic effects, although masculinisation of females has been observed in fish populations exposed to pulp-mill effluents or to anabolic steroids from cattle feedlot effluent. Laboratory studies have begun to address important issues such as that of mimicking and predicting the effects of complex mixtures of chemicals. These studies have served as exemplars for subsequent 'mixture' mammalian studies. Behavioural reproductive endpoints have been shown to be exquisitely sensitive compared with morphological endpoints, but there have been no convincing transgenerational studies examining actual fitness of the offspring. Although it is assumed that early life stages are more sensitive than adult stages, this has not been conclusively demonstrated in any fish species. Recent published data indicate population-level effects of exposure to EDCs on populations of fish examined in the field, but these are not catastrophic effects — populations appear to be sustainable. There is a notable lack of published data on age-class structure in exposed and non-exposed populations of fish and in linking reproductive impairment with ecological risk.

The worldwide decline in amphibian populations has drawn considerable attention, but the possible effects of EDCs on recruitment and abundance have not been investigated. Gonadal intersex, the classic indicator of oestrogenic ED, has been observed in frogs at contaminated sites, but causality remains uncertain. A number of effective bioassays for ED effects in amphibians have been developed during the last 10 years; they have been used to test a wide range of chemicals, usually those already shown to be active on other taxa. However, for many, no consensus yet exists on the pattern of effects. This is largely due to intra- and inter-species variation in basic developmental biology and susceptibility to EDCs. Like many wildlife species, the basic biology of the vast majority of species of interest is poorly understood. There is no doubt that amphibian habitats are contaminated by EDCs and that these

contaminants also exist in amphibian tissues. What is not clear is which, if any, contaminants contribute to amphibian population declines. This is a fertile area for research, as any chemical known to slow metamorphosis will undoubtedly affect amphibian survival.

Research into the effects of EDCs in reptiles has largely been driven by the progress of studies on alligator populations in Florida, and there is persuasive evidence for contaminant effects on reproductive function. Laboratory studies since 1996 have addressed ED effects in an increasing range of species, and have highlighted difficulties in reconciling field observations with results obtained under controlled conditions. As with amphibians, a greater understanding of the basic biology in crocodilians would help to determine normal variations in distinct endpoints and populations, and to distinguish it from abnormal ones.

The effects of DDT and its metabolites on bird reproduction were amongst the first effects of pollution on wildlife to be demonstrated. This and other persistent organic pollutants (POPs) can definitely cause skewed sex ratios, lowered fecundity and eggshell thinning, leading to widespread nesting failure. At the time of writing, however, the evidence for effects of ED in wild bird populations is fragmentary and does not always support the findings of the extensive body of laboratory-based studies that now exists. There is, nonetheless, no doubt that reproductive endocrine effects of contaminants have been noted in bird populations near wastewater treatment lagoons, and in other areas in connection with exposure to contaminants. The effects of anti-thyroid compounds and of goitrogens have received very little attention to date.

For invertebrates, the well-established case studies of tributyltin (TBT) effects in molluscs with severe impacts on communities and of insect growth regulators (IGRs) as intentionally designed EDCs have been complemented by a number of investigations carried out further field as well as in the laboratory. These studies have demonstrated the principle susceptibility of the often unique invertebrate endocrine systems for compounds described as EDCs in vertebrates, but they have likewise done so for other chemicals. Gaps in basic knowledge of invertebrate endocrinology remain the greatest obstacle to progress of ED research, although the consideration of invertebrates in research potentially offers a wealth of knowledge in understanding comparative and ecological aspects of ED.

2.2.3 Knowledge gaps and research priorities

Gaps

- Little targeted field monitoring to evaluate populations and communities in contaminated areas.
- Few studies on whether thyroid hormone, metabolic and neurodevelopmental disruption exists in wildlife populations in the field.
- Few studies link endocrine effects at the individual level to the population level.
- Few studies to bridge the gap between the many laboratory studies on ED effects to wildlife populations in the field.
- Little or no knowledge of endocrinology of many invertebrate species, and so no capacity to study ED or its potential in these groups.
- Very few studies on wild mammals, both for their own sake and as sentinels for human health.
- No studies address the ecological impacts of endocrine disrupters.
- No knowledge of the importance of ED relative to other threats to biodiversity.
- Few studies capitalise on the use of characterising effects in wildlife as sentinels for human health, for example, in urban areas versus more rural areas.

Research priorities

- Conduct properly designed semi-field studies to bridge the gap between existing field and laboratory findings.
- Evaluate the effects of mixtures that are representative of the exposure profiles in the natural environment with representative vertebrates and invertebrates.
- Conduct targeted field monitoring to evaluate the potential impact of EDCs on populations and communities in contaminated areas.
- Conduct ED studies with a broader range of invertebrate groups, considering both terrestrial and marine species.
- Continue to invest research effort in basic invertebrate endocrinology.

- Direct effort towards the characterisation of invertebrate hormone receptors in order to identify shared functionality between taxonomic groups to facilitate the development of extrapolation techniques for effects between species and for *in vitro* systems as screening tools.
- Develop new invertebrate tests with endocrine-regulated endpoints, and amend existing protocols.
- Optimise and accelerate the validation process for new regulatory tests.
- Utilise wild mammals in affected areas, e.g. wild rodents living in areas of biosolids application, or goats living on waste dumps.
- Start to address the question of ecological impacts of endocrine disrupters in, for example, causing declines in diversity and abundance of wildlife.

2.2.4 Implications for risk assessment and regulatory action

- Inclusion of currently under-represented systematic groups in laboratory studies will help to reduce the uncertainty in assessing potential effects of EDCs on wildlife.
- Carefully targeted monitoring programmes will determine whether and to what extent ED affects wildlife populations, and will facilitate an assessment of the impact of EDCs on global biodiversity.
- A taxonomically inclusive suite of tests will provide better coverage of invertebrate and vertebrate groups in freshwater, marine and terrestrial communities, and must therefore be developed to provide a sufficient protection level for these ecosystems.
- If in the natural environment, exposure to complex mixtures rather than to single agents is the normal situation, then appropriate predictive models are needed for all 'at risk' taxa.

2.3 Mechanisms and laboratory animal models

2.3.1 Introduction

Concerning mechanisms of actions of EDCs, emphasis at the time of the Weybridge meeting in 1996 was placed on oestrogenic compounds and especially on receptor-mediated effects. Furthermore, very little knowledge on mixture effects and low-dose effects of EDCs existed. Concerning *in vitro* assays, a limited range was available. An extensive research effort during the last 10 years has succeeded in providing important knowledge on and progress within these areas.

2.3.2 Progress summary

Mechanisms of endocrine disruption
A decade ago, the focus of both concern and action regarding EDCs was on hormone receptor agonists and antagonists, in particular oestrogen receptor (ER) agonists. The screening and evaluation systems for ER- or androgen receptor (AR)-mediated hormone activity continue to be widely used. However, in the past decade, the scope of EDCs has expanded considerably. The focus of research has now broadened to include EDCs that affect other hormone systems (e.g. thyroid), to EDCs with new modes of action (e.g. inhibitors of endogenous hormone production or metabolism), and to target tissues for EDCs other than those in the reproductive system (e.g. brain or cardiovascular system). There is also new additional evidence that some common environmental chemicals have the potential to exert multiple hormone-modifying effects (e.g. PCBs or azole fungicides).

The past decade has seen a considerable expansion of understanding of health disorders in humans and wildlife that have ED as a central feature, thus raising the possibility (still largely unproven) that EDCs could contribute aetiologically to these disorders. Reproductive-related disorders continue to dominate this area, but neurodevelopmental and thyroid hormone–mediated disorders have also joined the list. With the increasing prevalence of lifestyle-related disorders (e.g. obesity and type II diabetes) in which disruption of endogenous hormones plays a central role, a major task for the next decade will be to elucidate to which degree these disorders may be the result of exposure to EDCs.

Mixture effects of EDCs
The Weybridge workshop recognised the need for research on how mixtures of EDCs acted. EDC mixture work was in its infancy then, but within the last few years it has become an active research area and much has been learned. Mixture studies *in vitro* and *in vivo* on similarly acting oestrogens and anti-androgens mediating their effects via ERs or ARs have shown that mixture effects very often occur in an additive fashion. This also implies that adverse effects are often found for mixtures containing low doses of EDCs that do not individually exert any detectable effects. The dose addition approach seems to provide a solid basis for prediction of joint effects of multicomponent mixtures of receptor-mediated oestrogenic and anti-androgenic action. Mixture studies are now also considering how chemicals that disrupt common developmental pathways via diverse modes of action will interact.

Low-dose effects of EDCs
Some chemicals found in the environment act as EDCs and disrupt reproduction and development at very low doses in nanomolar (nM) to picomolar (pM) concentrations. Recent observations have revealed that pharmaceuticals can exist in the environment at physiological active concentrations. While most other anthropogenic EDCs appear relatively weak, considerable controversy exists over the existence, relevance and reproducibility of non-monotonic U or inverted U-shaped dose-response curves. In the last 10 years, research in this area has provided some indications for the existence of non-monotonic dose–response curves, but this has not resolved the scientific debate; in fact, if anything it has become more heated.

Screening and testing programmes for EDCs
In the last 10 years, many new *in vitro* assays for EDCs have been developed that greatly enhance the ability to study mechanisms of action and to screen large numbers of chemicals for hormone activity, in order to discriminate hormone agonists from antagonists. Quantitative structure–activity relationship (QSAR) models of EDC action using the data developed in these assays are being developed and evaluated. The OECD has undertaken the task to revise existing and to develop new guidelines for screening and testing of potential EDCs. Current screening efforts have focused on development and validation of assays to detect oestrogens, androgens and thyroid active substances. *In vitro* assays include binding assays, the more functional reporter gene assays and cell lines to assess steroidogenesis. *In vivo* assays are included as well, to detect oestrogens (uterotrophic assay) and androgens and anti-androgens (Hershberger assay). The US EPA also is validating assays of pubertal development in weanling male and female rats, since these models should be sensitive to all the above endocrine modes of action.

2.3.3 Knowledge gaps and research priorities

Gaps
- No *in vivo* assays that are currently part of the OECD framework for testing EDCs can identify mammary carcinogens.
- No models for testis germ-cell cancer exist.
- No single animal model for key features of prostate cancer in men.
- Need for correlations between *in vitro* and *in vivo* screens for TDS.
- Little knowledge of mechanisms of low-dose actions including epigenetics and non-genomic pathways.
- Little knowledge of effects in the low-dose range and how it differs from the higher dose range.

Research priorities
- Determine effects and modes of action of EDCs that act via novel pathways on targets not fully considered in the current strategy.
- Identify cellular and molecular mechanisms of EDC action (application of 'omics' technologies).
- Determine target tissue concentrations of EDCs during critical periods.
- Develop a framework for understanding how mixtures of EDCs from different classes (including mixtures containing large numbers of EDCs at low doses) with divergent modes of action behave in the developing animal.
- Replicate low-dose studies.
- Develop strategies to focus, streamline and speed up the implementation process of screening and testing.
- Optimise the validation process of tests.
- Develop high-throughput pre-screening (HTPS) and quantitative structure–activity

relationship (QSAR) methods for screening and prioritisation.

- Design of an optimal battery of screening tests for endocrine-disrupting effects.

- Current multigenerational assays need to be enhanced to include all the endpoints sensitive to key EDCs; multigenerational experimental designs need to be modified to achieve greater statistical results from fewer animals.

2.3.4 Implications for risk assessment and regulatory action

- New data are expected to reduce the uncertainty in extrapolating the effects from laboratory studies to humans and other species, since they may help determine how highly conserved the modes of action are and if the exposure levels in the laboratory are at relevant levels. Further insight into the mechanisms of actions of EDCs may also facilitate the design of a battery of tests for screening chemicals for endocrine-disrupting effects.

- If future research confirms the frequent findings of additive effects of EDCs, powerful tools for prospective risk assessment would become available. These tools could forge the way to make productive use of existing single chemical databases for the prediction of mixture effects. Thus, research within this field is expected to lead to science-based risk assessment and management decisions that protect humans and wildlife from the adverse effects of complex mixtures of EDCs.

- If solid evidence for low-dose effects is found, and it's agreed that they are adverse, the result is expected to be restricted use of the chemicals in question. Further regulatory agencies would have to re-evaluate the no- or low-observed-adverse-effect-levels (NOAELs-LOAELs) approach for risk assessment of EDCs, which may well lead to a paradigm shift in the risk assessment process.

- Validated screening procedures for EDCs that use a minimum number of animals will be applied.

- Reduced animal use and more accurate NOAELs or benchmark dose levels (BMDs) with better and updated testing protocols are expected. Results from efficient testing of large numbers of EDCs may lead to regulatory initiatives to minimise use of and exposure to problematic EDCs.

2.4 Exposure, risk and policy

2.4.1 Introduction

Ten years ago, a great deal of the data essential for chemical risk assessment procedures were not available. Relatively little was known about exposures, and test strategies for the identification of EDCs — an essential element of hazard characterisation — were under development. The need for taking mixture- and low-dose effects into consideration was acknowledged, but data useful for risk assessment were not available. Of considerable concern was that established risk assessment methods were incapable of anticipating the impact of EDCs. As a result, the effects of many EDCs on wildlife only became apparent after significant responses had already occurred. There are similar problems with conventional toxicity testing procedures used for evaluations of human health risks, and these procedures are now considered to be inadequate for the detection of EDCs.

2.4.2 Progress summary

Over the last 10 years it has become apparent that certain characteristics typical of EDCs make the application of traditional risk assessment processes difficult. Among these is the realisation that EDCs exert their effects during specific life stages (for example *in utero* or during puberty), but the health consequences of these interactions may become apparent only later in life. This delay in the manifestation of effects can span several decades, thus considerably complicating any attempts to establish causal relationships between exposure and effect.

Considerable progress has been made with understanding mixture effects of EDCs. It has become evident that the traditional chemical-by-chemical approach to risk assessment is inadequate when dealing with EDCs. The biological reality of combination effects from exposure to multiple agents at low doses highlights the potential for underestimating risks when mixture effects are not taken into account. This underlines the need to modify current risk assessment practice, if humans and the environment are to be protected adequately from multiple exposures to EDCs.

The realisation that every similarly acting EDC in a combination contributes to the overall mixture effect, in proportion to its potency and dose, undermines the traditional risk assessment paradigm of a threshold

dose below which a chemical fails to produce effects. Whether the individual doses are also effective on their own is immaterial in these situations, and consequently even doses below thresholds are of relevance. This is particularly important in situations where EDCs act together with endogenous hormones. Since there is already endogenous biological activity, the effect of even very small exposures is not zero. Whether the resulting additional effects are significant depends on the potency, level and number of EDCs. This also means that the effects of EDCs cannot be dismissed solely with the argument that their potency in relation to endogenous hormones is low. Thus, the idea of threshold doses begins to lose relevance when dealing with similarly acting EDC mixtures.

The mixture effect issues discussed above have important implications for EDC exposure assessment. If every similarly acting EDC can contribute to combination effects even at doses below effect thresholds, then it is essential to have comprehensive information about the array of EDCs that contribute to human and wildlife exposure. Although considerable progress has been made in identifying EDCs, our knowledge about the nature of EDCs relevant for human and wildlife exposure scenarios is still far from complete. At the heart of the problem lies the fact that EDCs need to be identified in terms of their biological effects, before chemical analytical methods can be brought to bear. To overcome these problems, toxicity identification and evaluation (TIE) schemes have been used. TIE combines chemical extraction and fractionation with *in vitro* bioassays for the identification of bioactive fractions, with the ultimate aim of pinpointing relevant EDCs. This approach has worked well with oestrogenic chemicals in the aquatic environment, but there is little experience with other *in vitro* assays or with human tissues or body fluids.

Exposure assessment schemes that directly address the mixtures issue by chemical analysis of a range of EDCs in one and the same sample are few and far between. Without a doubt, the lack of comprehensive EDC exposure assessments can be regarded as the bottleneck to better EDC risk assessment today. This challenge can only be met by adopting integrated approaches that take into account cumulative exposures through multiple routes, including food, inhalation and dermal uptake. Traditional exposure assessment schemes, with their focus on individual chemicals, are likely to fail to address the EDC issue. Progress has been made with exploiting biomonitoring approaches for exposure and risk assessment. Material from biobanks is invaluable in closing these data gaps.

Another basic premise of traditional risk assessment procedures is the idea that long-term effects at low levels of exposure can be anticipated by extrapolation from tests conducted at high doses with comparatively short exposure durations. With many chemicals that are devoid of ED effects, long-term effects normally fail to materialise at doses (or concentrations) below 0.1 % to 1 % of those obtained in short-term tests. However, this is not fulfilled with some EDCs, the synthetic oestrogen ethinyloestradiol (EE2) with an acute-to-chronic ratio of more than 1 million in fish serving as a striking example. For EDCs, there is little systematic information about the relation between dose and exposure duration. It will be important to know whether declines in effects due to a lowering of dose are counteracted by prolonged exposure duration.

Substantial progress has been made in understanding the importance of timing of EDC exposure during windows of increased vulnerability in certain life stages. The realisation that hormones are important developmental triggers represents another serious complication for exposure and risk assessment. If exposure assessment strategies are to capture potentially causative agents, monitoring has to take place during these windows of vulnerability, and not when disorders have become manifest. Monitoring during later life stages will produce a warped picture in terms of risk assessment, with risks likely to be overlooked. All these factors complicate enormously any attempts to establish a causal relation between exposure and effect, and there are serious doubts as to whether causality can ever be established for human populations under these circumstances. For these reasons, it is crucial to develop rational criteria for assessing the level of proof deemed necessary to trigger regulatory action.

It is widely recognised that current international testing schemes were not designed for EDCs, and that they have serious deficiencies in estimating EDCs' likely impact on human health and wildlife. In response to these problems, considerable efforts have been made by the OECD and other organisations (the Fund for the Replacement of Animals in Medical Experiments (FRAME) and the European Centre for the Validation of Alternative Methods (ECVAM)) to expand test development to include procedures responsive to EDCs. Despite all this progress, a serious dilemma relevant to hazard characterisation has remained largely unresolved: given that many EDCs act during specific developmental periods, an identification of the entire spectrum of EDC effects would require multigenerational studies and full life-cycle testing. However, due to the high costs and long duration of such tests, it is

unsustainable to subject every suspect chemical to such exhaustive testing. It is also untenable from an animal welfare point of view. In contrast, most of the available *in vitro* and *in vivo* screening assays can be conducted rapidly, but they only encapsulate interactions with the oestrogen, androgen and thyroid receptors and consequent downstream events, or interference with hormone synthesising or metabolising enzymes. Although positive test outcomes indicate the potential for ED, the relevance of these screening endpoints for risk assessment is often unclear. To complicate matters further, validated test systems for many human conditions of concern (for example, TDS) are still lacking.

The strategy followed by the OECD, for instance, in dealing with these difficulties is to promote the development of short-term assays that might act as triggers for more comprehensive testing. However, test guideline development and validation is a time-consuming process, and an adequate set of tests will not be available for many years. Meanwhile, concerns about EDCs are such that it would be unacceptable if the long time-frame for assay development would seriously delay or even block regulatory action. It is therefore necessary to develop a risk assessment and regulatory framework for dealing pragmatically with incomplete knowledge about EDCs; several interesting proposals for such frameworks have been made, with the following principles.

- Putative EDCs need to be dealt with on a case-by-case basis that requires expert judgement.

- Positive outcomes from *in vitro* and *in vivo* screening assays should generally trigger further testing in long-term assays, and *in vivo* testing is needed for more definite identifications of EDCs. However, this leaves the issue of false negative outcomes unresolved — something that leads to major problems in terms of overlooking EDCs.

- Because screening assays only capture certain aspects of endocrine action, negative outcomes cannot be taken as evidence for absence of endocrine activity by other mechanisms.

- Data from *in vivo* screening assays (e.g. uterotrophic assay or Hershberger assay) should be used for preliminary risk assessment purposes. This is justified because these assays indicate endocrine activity with relevance to humans and wildlife, and because the potency of EDCs in screening assays is often comparable with that in long-term studies. However, in view of their short-term nature, estimates from screening assays should be combined with larger-than-usual assessment factors for the conversion of NOAELs into human limit values (HLVs).

2.4.3 Research priorities

- Combinations of EDCs with differing modes of action should be assessed in terms of predictability of mixture effects. Ascertaining whether combination effects occur at doses below NOAELs, and whether dose addition can be used as the default model in such cases, are both of high regulatory relevance.

- Systematic research into the relationship between dose and duration of exposure are necessary to investigate the validity of extrapolations from short-term tests at high doses to long-term effects at low doses.

- Monitoring programmes focusing on chemical analyses of multiple EDCs in one and the same sample need to be implemented to support better exposure assessment. The suitability of a wide range of *in vitro* assays for TIE approaches should be investigated to aid the discovery of as yet unidentified EDCs. The application of TIE to samples of human origin requires urgent attention.

- Environmental and human biobanks should be set up to support analysis of time trends in pollution levels of EDCs. Analytical methods for polar substances need to be developed and validated.

- Much research is needed to develop biomonitoring strategies useful for exposure and risk assessment.

- Continued investment into research of the mechanisms underlying ED is urgently required to support the development of better testing and screening methods. Existing experimental models with demonstrated relevance to human disorders should be developed into test methods, and validated.

- The development of intelligent testing strategies that deal effectively with the limited relevance of screening methods for risk assessment purposes is of great importance and will require considerable research input to support the efforts of OECD and other supranational bodies.

- Rational criteria for dealing with knowledge gaps and uncertainty in regulatory

decision-making for EDCs need to be developed.

2.4.4 Implications for risk assessment and regulatory action

Progress made over the last 10 years of ED research has implications for risk assessment and regulatory action.

- As a first step in the direction of implementing better risk assessment and regulation for EDC mixtures, the idea of grouping EDCs according to suitable similarity criteria suggests itself, as is already common practice with the group-wise assessment of PCDDs and PCDFs. However, the challenge lies in finding workable criteria for grouping EDCs according to 'similar modes of action'. One suggestion would be to categorise EDCs according to their modes of action by adopting a phenomenological similarity criterion (e.g. 'all agents that disrupt male sexual development by inducing changes in anogenital distance'). Given the diversity of modes of action, too narrow a focus on molecular mechanisms might prove unworkable.

- Special consideration should be given to EDCs that act in concert with endogenous sex hormones. The assumption of threshold-mediated action does not apply in such situations, because every little dose quantum adds to an already existing effect. In such cases, it should be considered whether there are alternatives that can substitute such EDCs. If substitution for certain uses is possible, regulatory action should be taken without further risk assessment. Examples where this approach may be viable include oestrogenic agents such as ultraviolet (UV) filter substances, parabens, certain synthetic musks, and meat hormones.

Hazard classifications for EDCs within the Registration, Evaluation and Authorisation of Chemicals (REACH) framework need to take account of existing knowledge gaps about the full effect spectrum of EDCs. Because such information will not be available in the foreseeable future, hazard classifications have to make use of existing data, as follows.

- In cases where it is debated whether reproductive and developmental toxicity should be considered adverse, evidence from *in vitro* and *in vivo* screening can be used to demonstrate that chemicals work by a mechanism relevant to humans. This information can support upgrading from category 3 to category 2 and thereby trigger authorisation automatically.

- Positive results from *in vivo* screening assays such as the uterotrophic assay or the Hershberger assay may be used directly for classification. Considering that such chemicals are likely to be reproductive toxicants, placement in category 3 can be supported. In specific cases, it may be warranted to require authorisation for such category 3 chemicals until further test data become available to dispel concerns and aid decisions as to whether placement in categories 2 or 3 is justified.

- Chemicals where only *in vitro* data are available are not currently classified. But positive *in vitro* test outcomes should trigger entry into the EU list of potential EDCs. These substances should be prioritised for further testing.

2.5 Key conclusions, challenges and recommendations

Several key conclusions can be drawn from the research over the last 15 years, outlined below (and discussed in the plenary session at the Weybridge+10 meeting). Challenges and recommendations are also outlined:

1. ED is a real phenomenon likely affecting both human and wildlife populations globally, but a much better understanding of the role of chemicals as causal factors of a wider range of endocrine diseases and disorders is needed.

   Since the publication of the first Weybridge report, the term 'endocrine disruption' has become much more commonplace. The idea that chemical pollutants can interfere with the endocrine system in wildlife and humans is supported by numerous laboratory studies in which disruption of hormonally controlled processes (particularly reproduction and sexual development) have been seen in animals exposed to potential EDCs. These studies are mirrored by observational studies on domestic animals and wildlife species living in contaminated environments that clearly show ED. Studies of human endocrine diseases and disorders indicate an increased incidence and prevalence over the last few decades, particularly for childhood diseases. These incidences cannot be explained solely in terms of established risk factors. Considering the role of hormones in

reproduction and development and in adult and child physiology, one would predict that exposures to EDCs could play a causal role in the manifestation of these diseases. Pharmacological and occupational examples of ED in exposed people exist, and the environmental data are suggestive of chemically induced ED in the general population. A much better understanding of the role of exposure to environmental contaminants in endocrine disease risk in both humans and wildlife is needed.

2. Screening tests to detect endocrine disrupters exist but there are still inadequacies in some areas and these need to be addressed.

   As a result of immense scientific effort and media attention, the US EPA, the OECD, the US FDA, the European Commission and the European Food Safety Agency (EFSA) have developed a range of safety evaluations for EDCs, including *in vitro* and *in vivo* screens and computational approaches. Despite these efforts, however, none of the *in vivo* assays that are currently part of the OECD framework for testing EDCs can identify mammary carcinogens, and the following are lacking: animal models for testis germ-cell cancer, a single animal model for key features of prostate cancer in men, developmental neurotoxicity assays, and animal models for thyroid cancer.

   In wildlife, invertebrate tests for endocrine disrupters are hampered by lack of knowledge concerning the fundamental endocrinology of many of these animals and the mechanisms of action through which EDCs might operate.

3. Hormones (and hormone disrupters) are little molecules with big goals, many of which are not fully understood.

   Endocrine disrupters can interfere with the synthesis and/or action and/or transport and/or metabolism of oestrogens, androgens, thyroid hormone as well as with glucocorticoids, vitamin D, prolactin, insulin, vitamin A and others. They multitask and can interfere with the normal functioning of the hormonal system at multiple points to affect complex cellular processes such as growth and metabolism in various ways. For example, oestrogen exerts its effects by acting on at least two major receptor types, but other kinds of receptors mediate oestrogen action as well. The actions of a hormone in one cell type are almost always different from those in another cell type. Hormone receptors must interact with a variety of 'helper' proteins called 'co-regulators' in order to exert their effects; different combinations of co-regulators exist in different tissues of the body. Some chemicals have been shown to change the way the hormone receptor can interact with co-regulators, leading to complex effects on development and physiology. Endocrine disrupters (like hormones) are subject to feedback mechanisms that could result in non-monotonic dose responses.

   Some of the mechanisms via which endocrine disrupters could act are little studied. Examples are the inhibition of prostaglandin synthesis as another way of producing reproductive disorders, delay in the differentiation of stem cells (thus contributing to testis germ-cell cancer risk), and epigenetic effects and gene imprinting in irreversibly shaping the sensitivity of various tissues to endocrine cancers and mechanisms of thyroid carcinogenesis.

   Whilst the traditional endocrine glands (pituitary, thyroid, adrenals, and gonads) are well known, our increasing knowledge of endocrinology has led to the realisation that the heart, the fat tissue, intestines, and other tissues are also endocrine glands. Until recently, most work on endocrine disrupters was focused on the male and female reproductive systems and on brain development. Recently, endocrine disrupters that stimulate fat cell differentiation and influence insulin sensitivity and glucose tolerance have been discovered, thus opening up the possibility of a role for EDCs in obesity, type II diabetes and metabolic syndrome. We need to take a broader look at chemical contaminants to identify those that interfere with other systems.

4. Because of the abundance and diversity of possible targets within the endocrine system, we need more knowledge on what types of endocrine disrupters are most likely to be affecting humans and wildlife (which are most prevalent and potent?).

   It is important not to confuse the affinity of an endocrine disrupter for a hormone receptor with its potency. Differences in affinity do not always translate to differences in potency.

5. There is potential for an instrumental role of 'omics' technologies in the study of ED by integrating pathways of response and pattern recognition, and by finding early indicators of the potential for effects at individual or

population levels. Currently, however, we have little understanding of the predictive power of these technologies or of how they could be used to prioritise chemicals for more extensive screening.

6. Current evidence suggests that the risks posed by mixtures of endocrine disrupters are likely to be cumulative.

   There are multiple, often similarly acting, EDCs in our environment, the effects of which are usually additive. There are multiple routes of exposure and multiple mechanisms via which the same outcome can occur; concurrent and/or sequential exposures are common. Moreover, both the order and duration of exposures can influence the potential for interactions. In general, chemicals with the same mode of action are dose additive whilst those with different modes of action are response additive. We have little knowledge of the likelihood of non-additive interactions in humans exposed to multiple chemicals at environmental exposure concentrations, although there is clear evidence of non-additivity for many chemical exposures at high concentrations.

7. Considerable evidence suggests that low-dose effects of EDCs often cannot be predicted from high-dose testing. These 'low-dose effects' of EDCs have come under intense scrutiny as they oppose traditional toxicology paradigms. The different possible meanings of the term 'low dose' can cause confusion.

   Non-monotonic dose–response curves for endocrine disrupters, including 'U-shaped' or 'inverted-U-shaped' curves have been described, thus calling into question the appropriateness of assuming monotonicity as a basis for chemical risk assessments of EDCs. At very low doses, the effects of a chemical may actually become greater than they are at higher doses. This is a 'low-dose' effect and encompasses the concept of a non-monotonic dose–response relationship. An appreciation of the role of the endogenous hormones in influencing the shape of the dose-response curve is key to interpreting these types of response, as U-shaped dose/response curves may reflect changes in concentrations of endogenous hormones. Other enormous data gaps in this area include the pharmacokinetics of low doses of endocrine disrupters and mechanisms of low-dose actions, including epigenetics and non-genomic pathways. Another important issue is the lack of full dose-response curves in most studies, resulting in possible missed 'low-dose effects'.

8. Research over the last 15 years has illustrated that endocrine disrupters can have latent effects, occurring as a result of exposure during early life or even in past generations (epigenetic effects), thus highlighting the probability of a developmental basis for many endocrine diseases.

   This raises key questions concerning the ability to associate endocrine diseases with exposures: When should the exposure is measured? What are the critical windows in development when ED would most likely occur? What is the past history of exposure?

9. Population risks of endocrine disrupters undoubtedly occur, but studies in this area must consider the distribution of susceptibility, response, and dose amongst the population members. What is within the 'normal' for one person may not be so for another.

10. People are animals; ED clearly occurs in wildlife and in domestic and laboratory animals exposed to similar or the same doses of EDCs as those found in our environment, clear evidence that chemically induced ED in human populations is likely. This represents a failure in environmental protection that should be addressed. Notwithstanding this, the fact that even the wealthiest nations in the world do not have the immediate capacity to conduct the scientific research necessary to address this issue raises a big problem. Invoking the precautionary principle, limiting our exposure to such chemicals even before we have full scientific knowledge would seem a rational approach to take.

3 Background reviews

3.1 Human health effects

3.1.1 Male relevant endpoints

Testicular dysgenesis syndrome (TDS)
Male reproductive health problems (poor semen quality, testis cancer, hypospadias and cryptorchidism) are often found in association with one another (Walsh et al., 2009) and increasing evidence points to their common origin in foetal development (Skakkebaek et al., 2001). Poor semen quality may result from a contemporary exposure to a toxic drug or occupational exposure, and therefore it is not necessarily always a sign of a preceding developmental disorder. However, when cryptorchidism, hypospadias and/or testicular cancer are found in association with poor semen quality, the underlying problem is hypothesised to be a TDS caused by androgen insufficiency during the early development of the male reproductive organs.

The evidence associating increased risk of testis cancer with low fertility, hypospadias and/or cryptorchidism is increasing (Schnack et al., 2010) and the most recent evidence suggests a two- to three-fold increased risk of testis cancer among infertile men (Walsh et al., 2009; Peng et al., 2009) Animal experiments have shown that effects mimicking those of TDS can be induced by *in utero* exposure of rodent models to chemicals that interfere with androgen synthesis and/or action (e.g. Mahood et al., 2006). The TDS concept has helped to understand the possible origins of a whole spectrum of male reproductive health problems, and perhaps exposure–outcome studies should take this under consideration in their designs.

Semen quality
Male reproductive health has been a major focus of the research on EDCs from the beginning. Declining sperm counts and increasing incidence of testicular cancer were the early pointers to this research. Sperm counts, in particular, became a topic of debate when many researchers and industry representatives challenged the initial scientific findings indicating deterioration in semen quality. At the time of the Weybridge conference more than 10 years ago, there were studies showing a temporal decline in semen quality in some cities, e.g. Paris, France and Edinburgh, United Kingdom (Auger et al., 1995; Irvine et al., 1995), whereas some other centres found no trend, e.g. in Turku, Finland (Vierula et al., 1996), or even improvement, e.g. in New York, USA (Fisch et al., 1996; Paulsen et al., 1996).

Interestingly, in both Paris and Edinburgh, a birth cohort effect was observed: younger age cohorts' semen quality was not as good as that of the older age cohorts at the same age. It also seemed apparent that there were large geographical differences in semen quality. However, uncertainty remained as to whether these could have been due to technical variation in methods or selection of study subjects. Although the data are sparse, more recent studies in non-Western countries such as China, India (Adiga et al., 2008), and Tunisia (Feki et al., 2009) also indicate similar temporal declines in semen quality. Evidence of large geographical differences has also been further supported by standardised international studies in Europe and the USA, in which both the selection of participants and the methods were carefully harmonised and controlled by external quality assurance. These studies showed significant geographical differences in semen quality in fertile men in Edinburgh, Copenhagen (Denmark), Paris and Turku (Jorgensen et al., 2001); the lowest sperm counts were found in Copenhagen, whereas the Finnish men had the highest sperm counts. Similarly, in the American research *The Study for Future Families* (Swan et al., 2003) with a similar design to the European four-city study of

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partners of pregnant women (Jorgensen et al., 2001), significantly lower sperm concentration was found in men from Missouri as compared to those from New York, Minneapolis and Los Angeles in the USA.

Further criticism of the European studies regarding the bias of the study population (i.e. they were selected for fertility) led to further studies on unselected populations of 18-to-20-year-old men in Nordic countries (Jorgensen et al., 2002; Punab et al., 2002; Richthoff et al., 2002; Tsarev et al., 2005).

These studies revealed two main findings: (1) young men from the general population had only half of the sperm count of men with proven fertility in the same area, and (2) Finnish and Estonian men had significantly better semen quality than Danish and Norwegian men. Other studies from the Nordic areas indicated that the semen quality of Swedish men is better than that of Danish but worse than that of Finnish men (Richthoff et al., 2002). Interestingly, these findings closely mirror the statistics of testicular cancer: in areas with a low incidence of testicular cancer, semen quality appears to be consistently better than it does in areas of high incidence. Yet another country with a high testis cancer incidence, Germany, showed similar poor semen quality to Denmark and Norway (Paasch et al., 2008; Vierula et al., 1996).

Exposure to various EDCs may be a risk factor for male infertility, and a growing amount of evidence on semen quality versus exposure to various chemicals has emerged over the past decade. Overall, it would seem that there are strong and rather consistent indications that some pesticides such as DDT/DDE and organophosphates are associated with low sperm count, and that some PCBs are detrimental to sperm motility; these findings are derived from small pilot studies (e.g. Hauser et al., 2002, and Dallinga et al., 2002), but are also corroborated by bigger studies (e.g. Spano et al., 2005; Toft et al., 2006 and Richthoff et al., 2003, reviewed in Jurewicz et al., 2009).

In *The Study for Future Families*, for example (Swan et al., 2003), elevated urinary levels of the pesticides alachlor, atrazine, and diazinon in the nested case-control setting were associated with poor semen quality in American men in the four American cities (Swan et al., 2003; Swan, 2006). Moreover, serum concentrations of POPs such as PCB-153 and *p,p'*-DDE in Greenland, Poland, Sweden and Ukraine were associated with poor sperm motility (but not sperm concentration) and fecundity (in the case of *p,p'*-DDE). However, the likely causal agent could not be identified because of a strong correlation in serum concentration of a wide number of biopersistent pollutants (Toft et al., 2005 and 2006).

Several studies (Hauser et al., 2006; Wirth et al., 2008) report higher urinary concentrations of phthalate plasticisers (and/or their metabolites) in infertile than in fertile men (attending fertility clinics), indicating that exposure to these chemicals might be among the factors associated with declining sperm quality. These findings have not, however, been corroborated in studies of men from the general population, and so may be biased by the selection of sub-fertile and infertile men. It remains to be seen whether maternal exposure to phthalates has any bearing on semen quality in adult male offspring as suggested by the TDS hypothesis.

Only Mocarelli et al. (2008) has shown convincingly that dioxin exposure during infancy was associated with semen quality during adulthood in young men who had been victims of the Seveso dioxin accident in Italy. In another study of the same population, lactational exposure in addition to foetal exposure appeared crucial for the adverse effect (Mocarelli et al., 2011). In a different study, Swan et al. (2007) reported an inverse association between mothers' beef consumption and sperm concentration in their adult sons. It was hypothesised that this was due to increased exposure to oestrogenic hormones in beef *in utero*, particularly diethylstilbestrol, the synthetic oestrogen known to cause adverse effects on both the male and female reproductive tract during its early development. More longitudinal studies are needed to determine whether this is also true for other contaminants, either individually or collectively.

Testicular cancer

Increases in the incidence of testicular cancer have now continued for several decades, and it is particularly apparent in the countries with reliable cancer registries (Moller, 2001). Risk for testicular cancer increases with later year of birth, indicating a clear birth cohort effect (Richiardi and Akre, 2005; Liu et al., 2000; Zheng et al., 1996; McGlynn et al., 2003; Walschaerts et al., 2008; Holmes et al., 2008; Baade et al., 2008). Both Swedish and Danish immigration studies point to an environmental impact on the incidence by demonstrating the change in incidence rate from high to low and vice versa in second-generation immigrants from Denmark and Finland to Sweden, respectively, i.e. the risk appears to depend on the environment where the foetus develops (Hemminki and Li, 2002;

Myrup et al., 2008). This suspicion has been proved by studies showing a clear origin for testis cancer during stem cell development (Clark et al., 2007; Meyts et al., 2007).

Oestrogens are known to promote testicular germ-cell cancer (Bouskine et al., 2008) and indeed, foetal exposure to diethylstilbestrol is associated with a two-to-three–fold risk of testicular cancer as compared to control groups (for reference, see Toppari et al., 1996 and Strohsnitter et al., 2001). Environmental pollutants have also been associated with testicular cancer. In a case-control study of testicular cancer in Sweden, for example, mothers of cases had higher blood levels of PCB, polybrominated diphenyl ethers (PBDEs), and hexachlorobenzene (HCB) than control mothers, whereas *p,p'*-DDE levels did not differ (Hardell et al., 2006). Cases and controls themselves had similar levels of these toxicants. In general, the data to support an association between testicular cancer and exposure to EDCs are very scarce — not necessarily because the association is absent, but rather because of the long latency between exposure and effect, and the consequent requirement to measure maternal exposure rather than the exposure of the cases themselves.

Cryptorchidism

Trends in the incidence of cryptorchidism have been difficult to trace because of the differences in definitions and study techniques. However, there are some well-standardised studies that allow comparison in the United Kingdom (Scorer, 1964; John Radcliffe Hospital Study Group, 1992; Acerini et al., 2009). These studies indicate a significant increase in the incidence from the 1950s to the 1980s and further to the 2000s. Similar methodology was used in recent studies from Denmark, Finland and Lithuania (Boisen et al., 2004; Preiksa et al., 2005). These studies demonstrated a great regional difference in the incidence. The birth prevalence of cryptorchidism in Denmark was four times higher than in Finland, which is a similar difference as in the rate of testicular cancer between these countries. The incidence of cryptorchidism in Lithuania was higher than in Finland, but lower than in Denmark. There was also a four-fold increase in the incidence in Denmark as compared to figures from the late 1950s (Buemann et al., 1961). A Dutch study used a somewhat different design and there the incidence of cryptorchidism was found to be close to Lithuanian rates (Pierik et al., 2005).

The Finnish-Danish study group has reported exposure data from the case-cohort series that was collected in the cryptorchidism study. According to a Monte Carlo permutation, exposure to eight pesticides that were present in the highest concentrations in breast milk (a proxy for foetal exposure) was associated with an increased risk of cryptorchidism (Damgaard et al., 2006). Some studies have demonstrated an association of cryptorchidism with the parents' occupation: sons of gardeners or farmers had an increased risk of cryptorchidism or orchidopexy rate (Garcia-Rodriguez et al., 1996; Kristensen et al., 1997).Higher organochlorine concentrations were found in fat samples from cryptorchid boys than from control samples (Hosie et al., 2000), but DDE levels in mothers blood were not associated with the risk of cryptorchidism in offspring from another study (Longnecker et al., 2005). A positive correlation of the concentration of PBDEs in breast milk and cryptorchidism in the sons was also found in the Danish-Finish cohort study (Main et al., 2007). The concentrations of some phthalate esters in breast milk were associated with increased LH-testosterone ratio in sons in the Danish-Finnish cohort study, but there was no association with the cryptorchidism rate (Main et al., 2006). According to the authors, one possible explanation for an altered hormone ratio could had been the anti-androgenic nature of the exposure. In an American study, such an effect was also suggested when mothers' higher urinary phthalate concentrations were associated with shorter anogenital distances of the newborn boys (Swan et al., 2005). Anogenital distance can reflect androgenic action, and a smaller anogenital index can be used as a sign of exposure to an anti-androgenic influence. It is clear that no single exposure can be blamed for causing cryptorchidism, but it is possible that a mixture of exposures may contribute.

Hypospadias

Registry-based studies on testicular cancer are reliable, because the disease occurs at the time when men are otherwise at their healthiest age. The treatment is successful, and without it the men would die. Therefore all the cases are diagnosed and registered. The same cannot be said about hypospadias. Many of the cases remain undiagnosed, and even more will never be registered in the malformation registries (Toppari et al., 2001). However, some registries are very active in searching the cases through hospital discharge information, in addition to recording those that are reported by physicians directly.

Paulozzi reported a clear increase in the incidence of hypospadias in the USA (Paulozzi et al., 1997;

Paulozzi, 1999) between the 1970s and 1990s, whereafter it may have levelled off, as also suggested by Fisch et al. (2009), at least in the city of New York. Meanwhile, Jin et al. (2010) reports a more recent increasing trend in south-east China between 1993 and 2005. In Europe, increases seemed to occur earlier, but registry data were not very reliable (Toppari et al., 2001; Lund et al., 2006 and 2009). In a Dutch cohort study, the prevalence of hypospadias (0.7 %) was about five-fold higher than that reported by the malformation registry (Pierik et al., 2002). In a Danish-Finnish cohort study, there was a large difference in the prevalence between the countries (1 % vs 0.27 %) (Boisen et al., 2005; Virtanen et al., 2001). Thus, the Danish-Finnish differences in male reproductive health were evident in all aspects that were studied (testicular cancer, semen quality, cryptorchidism and hypospadias).

It is evident that the incidence of hypospadias varies remarkably between European countries and has shown an increasing trend, at least in the Netherlands and Denmark. The underlying reasons remain elusive and exposure-outcome studies are hampered by too few study subjects in either the exposure group or the case group.

Epidemiological studies of exposure-outcome relationships in hypospadias are often inconclusive because of the small number of either cases or exposure groups. First-born children have been shown to be at higher risk in many studies as compared with later birth order (Kallen, 2002). Two studies on diethylstilbestrol were suggestive of an increased risk in the exposed group (Vessey et al., 1983; Henderson et al., 1976), whereas exposure to oral contraceptives in early pregnancy was not associated with an increased risk, according to several studies (Storgaard et al., 2006). In the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort in England, the vegetarian diet of the mothers was associated with a five-fold risk of hypospadias (North and Golding, 2000), which could theoretically be linked to a co-exposure to herbicides and pesticides or (alternatively) excessive phytoestrogen load. No exposure data are available to corroborate or refute this hypothesis. Akre et al. (2008) reported that mothers' diet lacking fish and meat was associated with an increased risk of hypospadias. More recently, Ormond et al. (2009) reported no association between vegetarianism and an increased risk of hypospadias in children of mothers in south-east England, but instead found an increased risk with exposure to hairdressing products in the workplace. In Norway, an increased risk of hypospadias was found in farming families (Kristensen et al., 1997) whereas in Denmark such an association was not apparent (Weidner et al., 1998). In a case-control study, DDE levels in maternal serum were not associated with an increased risk of hypospadias (Longnecker et al., 2002), whereas a more recent case-control study showed that maternal exposure to HCB in Italian mothers was associated with increased risk of hypospadias in their offspring (Giordano et al., 2010).

Prostate

The prostate gland is a hormonally sensitive accessory sex gland with a secretion that nourishes and protects the sperm after ejaculation, and contributes to the ejaculate volume. In adult males, prostate volume is maintained by a balance between cell renewal and programmed cell death. This is regulated by hormones, mainly androgens.

Experimental animal data indicate that brief neonatal oestrogen exposure permanently imprints prostatic development and is associated with increased incidence of hyperplasia, dysplasia and adenocarcinoma with ageing (Raijfer and Coffey, 1979). Similarly, oestrogen administration to rats postnatally results in ventral prostate inflammation in adulthood (Prins, 1997). More recent studies in mice have shown that *in utero* exposure to small doses of the oestrogen-like chemical BPA or DES will result in an enlarged prostate with increased AR expression and a reduced sperm count (vom Saal et al., 1997; Timms et al., 2005). Higher doses of either compound resulted in the opposite effects on the prostate. Vom Saal's 'low-dose' results were refuted by Cagen et al. (1999) and by Ashby (1999).

Nonetheless, this oestrogen-induced altered morphogenesis of the prostate gland has been shown to predispose it to neoplastic development (Huang et al., 2004; Prins et al., 2007). For example, neonatal exposure to oestradiol and DES in rodent models increases the incidence of prostatic intraepithelial hyperplasias (PIN), which are preneoplastic lesions. Exposure to environmentally relevant doses of BPA did not result in the induction of PIN, but increased the sensitivity of the gland to develop PIN following a second hormonal exposure during adulthood (Ho et al., 2006). Based on these studies it has been hypothesised that the human prostate may be vulnerable to effects of small doses of EDCs with oestrogen-like activity. However, solid human data in this field are still lacking.

Prostate cancer is the most common male cancer in Western societies. The incidence of prostate cancer has increased in the last decades, something

attributed mainly to increased attention and better diagnosis. Prostate cancer is most often androgen-dependent and may be treated with endocrine modulators. In a few epidemiological studies, exposure to certain EDCs has been associated with increased incidences of prostate cancer, whereas other studies have indicated no such link (Bostwick et al., 2004). There is also an indication that exposure to phytoestrogens may increase the risk of prostate cancer (Wetherrill et al., 2005; Strom et al., 1999) although this is pending confirmation in larger studies with monitored exposures.

Several studies have indicated that exposure to certain pesticides is linked with an increased risk of prostate cancer (Morrison et al., 1993; Van Maele-Fabry et al., 2006). Notably, the US National Health and Nutrition Examination Survey (NHANES) data (1999–2004) recently showed a positive association between serum concentrations of OC pesticides (Hexachlorocyclohexane [HCH], trans-nonachlor and dieldrin) and the risk of prevalent prostate cancer in American men (Xu, 2010), and between HCH, *p,p'*-DDE and dieldrin in an Indian study of 70 newly diagnosed prostate cancer patients and 61 age-matched controls (Kumar et al., 2010). The most interesting study, perhaps, is a study in the French West Indies where the plasma concentrations of the oestrogenic insecticide, chlordecone (used extensively for more than 30 years), was correlated with significant increased risk of prostate cancer in 623 cases versus 671 controls (Multigner et al., 2010). Conversely, a Japanese case-control study of 14 203 men aged between 40 and 69 (Sawada et al., 2010) and a Canadian study comparing 79 incident cases of prostate cancer with 329 matched controls showed no association between organochlorines and prostate cancer in these populations (Aronson et al., 2010).

3.1.2 Female relevant endpoints

In contrast to the great deal of attention that has been given to a possible role of ED in the observed adverse trends in male reproductive health, less focus has been placed on possible effects of ED in women. The development of the foetus into a male is considered more sensitive to hormonal disruption than is the sexual differentiation of the female; the female sex differentiation is regarded as the default pathway of foetal development and therefore as largely autonomous. However, the function of female reproductive organs is also highly regulated by hormones, and thus may be the target of ED.

Breast cancer

Breast cancer is the most common cancer in women, with the highest incidence rates in North America, Australia and northern Europe. This incidence, which has increased steadily worldwide, cannot be explained by improved diagnosis alone. Genetic factors (including the major susceptibility genes BRCA1 and BRCA2) may account for up to 10 % of breast cancer cases in developed countries (McPherson et al., 2000), but their prevalence in the population is too low to explain the majority of breast cancer cases. The majority must therefore be a consequence of lifestyle and environmental exposures. This is evident from studies of migrants, which show quite clearly that incidence rises following migration from low- to high-incidence countries, particularly if this occurs at young ages and in the second and third generation of immigrants.

Well-known risk factors for female breast cancer are younger age at menarche, older age at menopause, being nulliparous, and older age at first birth; higher parity, longer lactation and bilateral ovariectomy are protective, suggesting that longer lifetime exposure to oestrogen and progesterone is a risk for this cancer (reviewed in Bernstein et al., 2002). Pharmaceutical hormones similarly have been shown to affect risk as hormone replacement therapy for postmenopausal women increases breast cancer risk (Weiss et al., 2002). Factors that affect onset of puberty, female fertility, and age at menopause may thus be expected to also indirectly influence breast cancer risk.

However, EDCs may also have direct effects on the mammary tissue. Development of the mammary gland can be divided into three critical periods of growth: foetal development, adolescence, and pregnancy and lactation, all which are regulated by hormones and growth factors. Before birth, the mammary gland is in a partially undifferentiated state, and the terminal end buds (undifferentiated structures in the mammary gland) remain until puberty, when they undergo proliferation and differentiation into alveolar lobes. During pregnancy, when the mammary gland prepares for lactation, the lobular structures attain their most differentiated state.

Throughout life, stem cells that maintain their renewal cycle can be found in the human breast. These stem cells have been speculated to be the origin of at least a subset of human breast cancers (Wicha et al., 2006). The protective effect of early age of first birth and the increased risk of breast cancer among atomic bomb survivors exposed before the

age of 20 compared to exposure at older ages (Land et al., 2003) indicate that the mammary gland may be more susceptible to carcinogenic influences before the final differentiation during pregnancy.

In 1990, Trichopoulos suggested that breast cancer might originate *in utero* (Trichopoulos, 1990). His hypothesis was based on the following arguments: (1) oestrogen exposure is thought to be related to risk of adult disease; (2) exposures that act postnatally can also act prenatally; (3) oestrogens are 10 times higher during pregnancy than at other times in a woman's life; and (4) pregnancy oestrogens vary widely across individuals and may be related to exogenous factors. Subsequently, a number of studies have looked for associations between factors associated with *in utero* development and future risk of breast cancer (Potischman and Troisi, 1999; Michels and Xue, 2006; Okasha et al., 2003)

Evidence for associations between having been born of a pre-eclamptic or twin pregnancy and future breast cancer was found, with preeclampsia being protective and twin pregnancy increasing risk (reviewed in Potischman and Troisi, 1999). As pre-eclamptic pregnancies are associated with decreased oestrogen levels and twin pregnancies are associated with increased oestrogen levels, these findings are in line with the hypothesis that variation in pregnancy oestrogen may affect future breast cancer risk. Size at birth has been shown to be positively associated with risk of breast cancer (Michels et al., 1996; Vatten et al., 2005) especially for premenopausal breast cancer. Some studies only included birth weight, but Vatten et al. found that both birth weight and birth length was associated with increased future risk of breast cancer (Vatten et al., 2005). The association with birth length was stronger and after mutual adjustment for birth weight and birth length, only the birth length remained significantly associated.

The mechanism underlying the association between size at birth and the risk of future breast cancer remains elusive. But size at birth is likely a marker for high intrauterine levels of growth factors. These growth factors may also affect the number of susceptible stem cells in the mammary gland. In this respect, it is interesting that size at birth also is positively associated with mammographic breast density (Cerhan et al., 2005), as increased mammographic breast density is a major risk factor for breast cancer. There was also a negative trend between preeclampsia and mammographic breast density, although this did not reach statistical significance.

However, these findings suggest that mammographic density may be a marker of *in utero* effects on the mammary gland. Thus, elevated levels of natural oestrogens during critical time periods are associated with increased breast cancer risks later in life. Very recently, evidence became available that synthetic oestrogens can have similar effects. Between 1953 and 1971, approximately 300 000 women in the United Kingdom alone used DES, an oestrogenic drug, to avoid miscarriages. Women whose mothers took DES face twice the normal breast cancer risk (Palmer et al., 2006). The risk is expected to grow further as these 'DES daughters' reach menopausal age.

Epidemiological studies of the association between exposure to specific chemicals and human breast cancer are available. Occupational studies provide fairly consistent evidence that exposure to benzene, organic solvents and polycyclic aromatic hydrocarbons (PAHs) is associated with increased breast cancer risk (reviewed in Brody et al., 2003). In relation to the industrial accident in Seveso, a 2-fold increase in breast cancer risk among women with a 10-fold increase in serum level of dioxin was observed (Warner et al., 2002). More recently, Lopez-Carillo et al. (2010) showed that exposure to diethyl phthalate (measured in urine) may be associated with increased risk of breast cancer in Northern Mexican women.

Most of the existing population-based studies have measured chemical residues at the time of diagnosis or interview, assuming that these recent measures are proxies for historical exposures. This approach limits the investigations to persistent and lipophilic compounds that can be measured in adipose tissue and blood years after the exposure.

The most studied compounds are DDT and PCBs, but chlordane and dieldrin have also been studied. Associations between DDE (the primary metabolite of DDT), chlordane, dieldrin, and some PCB congeners and elevated risk of breast cancer have been observed in some studies, but not in others (reviewed in Brody et al., 2003 and Lopez-Cervantes et al., 2004). Some of these discrepancies are presumably due to the long time-span between the actual harmful exposure event and the diagnosis of the breast cancer. The study by Hoyer et al. that showed a significant positive association between breast cancer and blood levels of dieldrin had access to blood samples drawn in the 1970s and early 1980s from women followed up during a 17-year period (Hoyer et al., 1998). One of the possible explanations for their positive findings compared with the contradictory findings in the other studies might be that blood measures were taken

closer to the time of dieldrin use, which ended in the late 1970s.

Similarly, Cohn et al. (2007) also showed a positive association between serum DDT obtained from women during the 1959-to-1967 period (of peak DDT use) and breast cancer risk. Subjects were members of the Child Health and Development Studies, Oakland, California who provided blood samples 1 to 3 days after giving birth (mean age = 26 years). Cases (n = 129) developed breast cancer before the age of 50 years and controls were matched to cases on birth year (n = 129). High levels of serum DDT predicted a 5-fold increased risk of breast cancer among women born after 1931 (i.e. under 14 years of age in 1945 when DDT came into widespread use). Women who were not exposed to DDT before 14 years of age showed no association between DDT and breast cancer.

Chemicals such as DDT, DDE, or PCBs do not act in isolation in a woman's body, but in concert with the natural oestrogens and a large number of other hormonally active chemicals. These include, for example, chemicals released during the preparation of food (e.g. during the grilling of meat), and a growing plethora of man-made chemicals found as environmental pollutants (PCDDs, PCDFs and certain pesticides), used in cosmetics (i.e. antioxidants, UV filter agents, and some synthetic fragrances), or which leak from plastics (e.g. BPA, nonylphenol), as well as plant-derived oestrogens in certain foods. The oestrogenicity of many of these chemicals is considerably lower than that of natural or pharmaceutical oestrogens. Nevertheless, laboratory experiments have shown that a sufficient number of such chemicals can add to the effects of natural oestrogens, even when they are present at levels that individually do not produce measurable effects (Rajapakse et al., 2004).

This work highlights that the focus of the previous human studies attempting to investigate the effects of chemicals on breast cancer was problematic. Instead of concentrating on a few, arbitrarily selected substances, the entirety of hormonally active chemicals must be considered. A recent study among Spanish women demonstrated that breast cancer risk was associated with the body burden of all oestrogenic chemicals, excluding the natural hormones (Ibarluzea et al., 2004). Additionally, more recent studies (Valeron et al., 2009) have shown that environmentally relevant mixtures of oestrogenic chemicals at concentrations close to those detected in human tissues can sharply upregulate the expression of genes involved in the induction of transformation processes in human breast cells. This is the first evidence that chemicals in our environment, with oestrogenic properties that are accidental and not just natural hormones or pharmaceutical oestrogens, may contribute to the development of breast cancer, but further evidence is needed to substantiate this link.

However, the timing of the exposure in relation to sensitive developmental windows should also be taken into consideration in the data evaluation. The indications that breast cancer may originate early in life makes the presumed large time-span between the (first) adverse exposure event and the manifestation of the cancer an enormous challenge for the investigation of exposure-outcome relationships. In this respect, early markers of effects on mammary gland tissue like the mammographic breast density may prove valuable.

Uterine pathology

Endometriosis (defined as the presence of endometrial tissues outside the uterine cavity) and uterine leiomyomas (caused by benign proliferation of uterine smooth muscle cells; fibroids) are common disorders affecting 1 in 7 women of reproductive age (Missmer and Cramer, 2003) and 20 % to 25 % of premenopausal women, respectively. Clinically, the disorders present with irregular bleedings, abdominal pain and infertility. Both diseases are oestrogen-dependent, and it has been hypothesised that oestrogenic compounds from various sources, including environment, could play a role in their pathogenesis (McLachlan et al., 2006).

Experimental studies and treatment protocols in human medicine clearly show that the course of endometriosis can be modified by oestrogens. Some human studies have shown an association between exposure to PCBs and dioxins, and endometriosis (Buck et al., 2006; Birnbaum and Cummings, 2002; Tsukino et al., 2005; Rier and Foster, 2003; Rier, 2002), but others, including a follow-up study from Seveso investigating women exposed to high levels of TCDD, have failed to show a statistically significant association (Eskenazi et al., 2002).

Major differences in study design, analytical conditions and the number and kinds of congeners measured render comparisons between the different studies difficult (Anger and Foster, 2008; Heilier et al., 2008). The major problem is that some PCBs have a dioxin-like mechanism of action whilst others are oestrogenic and/or anti-androgenic. A relatively recent study, the largest in Italy, attempted to solve these problems by measuring total oestrogen and dioxin equivalents in serum (using *in vitro* reporter

gene assay) as well as individual PCB and dioxin congeners.

The results showed an increased risk of endometriosis with exposure to all of the non-dioxin-like PCB congeners, and with their summed concentrations, as well as with *p,p'*-DDE. Moreover, the odds ratios (ORs) increased with increased concentrations of PCBs. Conversely, no correlation between endometriosis risk and concentrations of dioxin-like PCBs or with total dioxin toxic equivalent (TEQ) was found. This study and its pilots (Porpora et al., 2006; Quaranta et al., 2006) support a role of some PCBs and *p,p'*-DDE in the pathology of endometriosis in humans in Italy, and refute the existence of an association between serum dioxin concentration and this disease. A recent comprehensive review of the literature confirms the absence of a link with dioxin exposure (Guo et al., 2009).

In contrast to the dioxins, there is increasing concern that phthalates, used as plasticisers for polyvinyl chloride (PVC) since the 1930s, may have negative effects on human fertility through their effects on steroid hormone synthesis. Cross-sectional epidemiological studies over the last decade (Cobellis et al., 2003; Luisi et al., 2006; Reddy et al., 2006a and 2006b; Itoh et al., 2009) have revealed an association between endometriosis and leiomyomas risk, and exposure to some phthalate plasticisers. Most of thesestudies were, however, small (with between 5 and 57 cases), adjusted for few, if any, potential confounders and often recruited controls from clinical settings. Moreover, most of these studies relied on serum measures of phthalates and therefore, could be prone to contamination from laboratory plastics. A single larger study of 1 227 women participating in the NHANES from 1999 to 2004 and of 838 women from the NHANES from 2001 to 2004, and using urinary concentrations of phthalate metabolites rather than the parent compounds, revealed positive associations with the metabolite of dibutyl phthalate (MBP) (Weuve et al., 2010). This study is consistent with earlier studies in experimental animals showing clearly that exposure to phthalates is associated with smaller preovulatory follicles, anovulation or delayed ovulation, longer oestrous cycles, decreased synthesis of oestradiol, decreased serum progesterone and increased serum follicle-stimulating hormone (FSH) (Davis et al., 1994a and 1994b).

Ovarian pathology

PCOS is a heterogeneous condition, but the most widely accepted definition is the association of clinical and/or biochemical evidence of androgen excess with chronic anovulation. It is often associated with obesity and the metabolic syndrome. The presence of polycystic ovaries is necessary for the development of the syndrome, but not all women with polycystic ovaries have PCOS. Overall, 6 % to 8 % of reproductive-aged women suffer from PCOS, making this disorder one of the most common endocrine abnormalities (Azziz et al., 2004). Although more women are being diagnosed with PCOS, it is uncertain whether this represents an increase in incidence. It is more likely due to more awareness and better diagnosis.

The aetiology of PCOS remains unclear, but it has been suggested that genetically determined hypersecretion of androgens by the ovary during puberty or likely during foetal development is involved, resulting in disruption of the hypothalamic-pituitary axis such that excess LH is secreted (Abbott et al., 2002). However, animal studies show that exposure of the foetus to excess androgens in the maternal circulation results in many of the features of PCOS as the animal reach adolescence (Eisner et al., 2002).

Thus a role of exogenous factors leading to excess androgen action during the development/imprinting of the hypothalamic-pituitary-ovarian axis cannot be excluded. To date, only one study links BPA in women's serum with increased risk of PCOS (Takeuchi et al., 2004). However, it is possible that the elevated BPA is a consequence, and not a cause, of PCOS; women with PCOS have high plasma testosterone and elevated T concentrations are known to decrease the clearance of BPA from the plasma (Takeuchi et al., 2006).

Ovarian cancer shares some of the same risk factors as breast cancer, including a protective role for pregnancy and lactation and increased risk at early age at menarche, late age at menopause, and hormone replacement therapy. Ovarian cancer is as such also a hormone-related cancer, and a distorted hormone balance may be involved in the development and progression of the cancer. There are very few, if any studies on ovarian cancer in relation to EDC exposure. A recent study, however, appeared to suggest that use of nonsteroidal anti-inflammatory drugs (NSAIDs) was associated with an increased risk of developing this cancer (Wu et al., 2009).

3.1.3 Puberty

Puberty is under endocrine control, and EDCs may affect the timing and tempo of pubertal development

(Teilmann et al., 2002). EDCs with strong hormonal activity (oestrogens) can induce peripheral puberty, whereas some weaker EDs may modulate the timing of central puberty. Most of the adverse effects of EDCs are related to precocious puberty in girls, most often premature thelarche. Numerous case reports have been reported on isosexual precocious puberty in girls and heterosexual precocious puberty in boys after accidental oestrogen exposure through cosmetics, food or pharmaceuticals (Partsch and Sippell, 2001; Henley et al., 2007). Recent trends in age at breast development in girls (Euling et al., 2008 in the USA; Castellino et al., 2005 in Italy; Semiz et al., 2008 in Turkey; Aksglaede et al., 2009b and Juul et al., 2006 in Denmark) have alerted paediatricians, who question whether this could have been caused by exposure to EDCs (Parent et al., 2003).

Clusters of cases with premature puberty have been found in many countries. The largest epidemic occurred in Puerto Rico in the 1970s (Comas, 1982). Environmental factors, including maternal factors, dietary habits, phytoestrogens, plasticisers and pesticides were studied as possible causes. Oestrogenic contamination of food, particularly poultry, could have been involved, because elevated oestrogenic activity was identified in food samples, and withdrawal of local chicken, milk, and beef from the diet caused involution of the breast tissue within 2 to 6 months in 58 % of the patients (Saenz de Rodriguez et al., 1985). Smaller epidemics were reported in Italy and Bahrain, where food contamination was also suspected (e.g. Teilmann et al., 2002).

Prevalence of precocious puberty is exceptionally high in children who have immigrated to Europe (Proos et al., 1992; Virdis et al., 1998; Krstevska-Konstantinova et al., 2001). These children typically have idiopathic central precocious puberty and their menarcheal age is significantly earlier than that of non-immigrant girls or co-patriots staying in the home country. The reason for this precocity has not been identified, but change of the environment is certainly the key for search of aetiology. High levels of persistent DDT derivative *p,p'*-DDE was found in 26 immigrant girls with precocious puberty, whereas only 2 of 15 Belgian patients had detectable serum DDE concentration (Krstevska-Konstantinova et al., 2001). The DDE levels correlated positively with the age of immigration and negatively with the time since immigration, suggesting that the source of contamination was in the home country (Parent et al., 2003).

o,p'-DDT has a weak oestrogenic activity, whereas *p,p'*-DDE is an anti-androgen. These findings formed the basis of a hypothesis that an early exposure to oestrogenic DDT would act peripherally to stimulate development of secondary characteristics, inhibit pituitary gonadotrophin secretion and stimulate hypothalamus. Secondary precocious puberty would start when the exposure disappears and the hypothalamic-pituitary axis gets activated, as occurs often in congenital adrenal hyperplasia when proper substitution therapy is commenced (Parent et al., 2003). Animal experiments have partly supported the hypothesis of the exposure-withdrawal effect as a signal for early onset of puberty, although the long half-life of DDT makes the sudden decline in DDT unlikely (Rasier et al., 2006).

In the USA, exposure to DDT of fish-eating mothers and their controls was measured in Michigan, and the timing of puberty in 151 daughters was studied (Vasiliu et al., 2004). Foetal exposure to high levels of DDE was associated with an advanced age at menarche. In North Carolina, no significant exposure-outcome associations between DDE and pubertal timing were found in the infant feeding study of 316 girls and 278 boys (Gladen et al., 2000).

Accidental animal food contamination with polybrominated biphenyl (PBB) in Michigan caused extensive exposure through meat and dairy products from contaminated cows. Perinatal exposure of children was extrapolated from PBB levels in serum of their mothers some years after exposure. Highly exposed girls had an earlier age at menarche and earlier pubic hair development than those who were less exposed through breastfeeding; no differences were found in breast development. The information was collected by self-reporting, and this may have caused more inaccuracy and variation in data on timing of breast development than on other pubertal signs (Blanck et al., 2000).

High lead levels in blood were associated to a delayed age at menarche and delayed pubic hair development, according to two studies that were based on the NHANES III in the USA (Selevan et al., 2003; Wu et al., 2003). Breast development was also found to be delayed in one of the studies (Selevan et al., 2003).

Effects of PCBs on the timing of puberty were studied in Belgian children from rural and urban areas (Den Hond et al., 2002). No association of PCB levels to pubertal development was observed in girls, whereas a significant delay of puberty was found in urban areas and in association with high PCB levels in boys. In the North Carolina Infant Feeding Study, no association of PCB exposure to the timing of puberty was found (Gladen et al., 2000). Two studies from

the Great Lake area, Michigan, found no correlation of PCB exposure to self-reported timing of puberty in girls (Blanck et al., 2000; Vasiliu et al., 2004). In a boy cohort from the Faeroe Islands, no association of PCB exposure to the timing of puberty was found (Mol et al., 2002). In the Yucheng accident in Taiwan, 55 boys who were exposed to high PCB and polychlorinated dibenzofuran (PCDF) levels were found to have shorter penile length than the control boys at the same age, suggesting pubertal delay (Guo et al., 2004).

Children are exposed to phthalate plasticisers; the epidemic of early breast development in Puerto Rico was followed by many studies on putative endocrine disrupters including phthalates (Colon et al., 2000). Phthalates were linked to gynaecomastia, because two thirds of 41 girls versus 14 % of 35 control subjects had measurable phthalate levels in serum. These phthalate levels were, however, subject to criticism owing to technical inconsistencies and possible contamination (McKee et al., 2004)

Epidemiological studies on the effects of EDCs on timing of puberty are summarised in Table 3.1.

3.1.4 Possible effects on non-reproductive organs

It is recognised that not only the reproductive system but also the function and/or regulation of non-reproductive organs may be the targets for ED.

Thyroid function

Undiagnosed clinical hypothyroidism (low thyroxine (T_4) with elevated thyroid stimulating hormone (TSH)) is relatively common in women of reproductive age (approximately 0.3 %), but untreated subclinical hypothyroidism (elevated TSH without T_4 below the reference range) and hypothyroxinaemia (low T_4 with normal TSH) are much more common (occurring in about 3.0 % to 3.5 % of women of reproductive age). Minor changes in thyroid hormone levels due to exposure to EDCs are possible and may not be detected in small cross-sectional studies, where the expected large inter-individual variations may camouflage real population differences associated with exposure.

The hypothalamic-pituitary-thyroid (HPT) hormone axis is the most studied non-reproductive hormone system with regard to effects of environmental chemicals. Growth and development in foetal life and childhood is highly dependent on normal levels of thyroid hormones. Particularly during gestation, normal levels of thyroid hormones are crucial for the development of the central nervous system. During this critical phase, even subtle changes in thyroid hormone levels may lead to irreversible developmental deficiencies, whereas the same scale of changes in the adult may only lead to subclinical hypothyroidism. Perinatally, thyroid hormones also play a role in Sertoli cell proliferation and differentiation, and thereby also may influence future male fertility.

The mechanisms involved in thyroid hormone homeostasis are numerous. Pollutants can interfere with iodine uptake to the thyroid follicular cells (Tonacchera et al., 2004; Breous et al., 2005; Schmutzler et al., 2004) disrupt thyroid hormone transport and metabolism by binding to thyroid hormone receptors and/or transport proteins (Meerts et al., 2000; Purkey et al., 2004; Kudo and Yamauchi, 2005; Ishihara et al., 2003) or change

Table 3.1 Association of exposure to EDCs with timing of puberty

Endocrine disrupter	Association with timing of puberty	Reference
DDT, DDE	High levels in immigrants with central precocious puberty	Krstevska-Konstantinova et al., 2001
	High DDE levels with early menarche	Vasiliu et al., 2004
	No associations	Gladen et al., 2000
PBB	High levels with early menarche	Blanck et al., 2000
PCB	High levels with delay in boys,no association in girls	Den Hond et al., 2002
	No association in boys or girls	Gladen et al., 2000
	No association in boys	Mol et al., 2002
Lead	High levels with delay in girls	Selevan et al., 2003; Wu et al., 2003

Note: DDT: 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane;
DDE: 1,1-dichloro-2,2-bis(4-chlorophenyl) ethylene;
PCB: Polychlorinated biphenyl;
PBB: Polybrominated biphenyl.

thyroid hormone–stimulated gene transcription (Zoeller et al., 2005; Li et al., 2010).

Several human epidemiological studies on the effects of environmental chemicals on thyroid function have been performed (reviewed in Eisner et al., 2002 and Bloom et al., 2003). The most studied compounds are PCBs. PCBs are known neurotoxicants in humans when exposures occur prenatally. Recent studies in particular indicate that PCB exposures are linked to a reduction in IQ as well as a reduction in response inhibition; moreover, this relationship is stronger in children with a small genu of the corpus callosum as measured by magnetic resonance imaging (MRI).

Some authors propose that these effects may be related to the ability of PCBs to interfere with thyroid hormone signalling. Associations between PCB exposure and reduced thyroid hormone levels (triiodothyronine (T_3), T_4) and/or enhanced levels of TSH in pregnant women have been reported in several studies (Ribas-Fito et al., 2003; Takser et al., 2005; Chevrier et al., 2008 and 2010), but not in others (Wilhelm et al., 2006 and 2008).

Overall, the studies indicate that maternal thyroid function, important for the neurological development of the foetus, may be altered by PCBs (e.g. Langer et al., 2009). Similarly, several studies (Darnerud et al., 2010; Herbstman et al., 2008; Chevier et al., 2007) have reported negative correlations between maternal exposure to PCBs and dioxins and thyroid hormones in infant blood samples (e.g. Koopman-Esseboom et al., 1994). Several other studies did not report these associations (Wilhelm, 2008; Dallaire et al., 2008; Longnecker et al., 2000; Matsuura et al., 2001). The inconsistencies are thought to be due to difficulties in obtaining sufficiently large populations as well as the fact that serum levels, especially of TSH, change dramatically during early life (particularly during the first few days after birth).

Longer-term effects of perinatal exposure may also persist in older children. Several studies found negative correlations between PCB levels in serum and thyroid hormone levels at the ages of 4 years, 7 to 10 years (Osius et al., 1999) and 10 to 15 years (Schell et al., 2004). The thyroid volume is another endpoint for thyroid function but is rarely used in human toxicological studies. However, in a study of adults from a PCB-polluted area, the thyroid volume (assessed by ultrasound) was found to be significantly larger than in non-exposed subjects, and the largest thyroid volumes were clustered among the subjects with PCB levels above the 95 % percentile (Langer et al., 2003).

Brominated flame retardants are another group of contaminants that have gained interest as potential EDCs. These include tetrabromobisphenol A (TBBPA), polybrominated diphenyl ethers (PBDEs), and polybrominated biphenyl. TBBPA and PBDEs show an even closer structural relationship to T_4 than PCBs, and in animal studies, PBDEs have been shown to reduce circulating levels of thyroid hormones. Until recently, only a few small human studies on flame retardants and thyroid function existed, and their results were not conclusive (Hagmar et al., 2001; Julander et al., 2005; Mazdai et al., 2003). Recently, however, a large study of consumers of fish from the Great Lakes on the Canada–United States border reported negative associations between concentrations of PBDE congeners in serum and serum levels of T_3 and TSH, as well as a positive relation to T_4 (Turyk et al., 2008). Overall, the results are conflicting.

In animal studies, histopathological changes have been observed after di(2-ethylhexyl)phthalate (DEHP) exposure corresponding to hyperactivity of the thyroid gland whereas exposure to di-*n*-butyl phthalate (DBP) decreased T_3 and T_4 in a dose-dependent manner. Human studies on thyroid effects are scarce.

In a follow-up examination of 19 adolescents, who were exposed to large amounts of DEHP due to invasive treatment in the neonatal period, normal thyroid hormone levels were found (Rais-Bahrami et al., 2004). More recently, however, significant negative associations were found between DBP levels in the urine of 76 pregnant women and both total and free T_4 (FT_4) (Huang et al., 2007). Likewise, negative associations have been found between DEHP exposure and FT_4 and T_3 in adult men (Meeker et al., 2007). Changes in thyroid hormone levels as a result of exposure to environmental chemicals may be transient, but nevertheless have permanent effects if the changes occur at critical developmental time points.

Two studies indicate that exposure to perfluorinated chemicals may interfere with human thyroid function. A sub-study of the American NHANES study found that women with high levels of perfluorooctane sulfonate (PFOS) were more likely to report current thyroid disease (Melzer et al., 2010). Similarly, a large study of 506 employees in a perfluorinated chemical (PFC) manufacturing company showed negative associations between PFOA exposure and FT_4 (Olsen and Zobel, 2007).

It is important to take into consideration that epidemiological studies are uniformly based on the

assumption that environmental chemicals interfere with thyroid hormone signalling solely by causing measureable changes in serum thyroid hormones. This assumption is clearly refuted in the animal literature, and continuing to pursue this assumption undermines the position of risk assessment agencies trying to identify thyroid disrupters as well as understand their adverse effects. Thus, a key weakness in this field is that we have no biomarkers of exposure effects on thyroid hormone action in tissues.

Neurodevelopment

It is very well established that thyroid hormones are of special importance in the development of the brain. Numerous *in vitro* and animal studies have shown that the absence of thyroid hormones reduces neuronal growth and differentiation in the cerebral cortex, hippocampus and cerebellum. This is of special importance in foetal life: development of the brain *in utero* is dependent on normal levels of thyroid hormones, and during the first trimester of gestation, the foetus is dependent on transplacental supply of maternal T4, and consequently on the ability of the maternal thyroid gland to increase hormone production during pregnancy in order to meet the needs of both foetus and mother.

As already described, interference with thyroid hormone homeostasis can take place on many different levels of the HPT axis, and may result in alterations of thyroid hormones available for the TH receptors. In cases of markedly reduced hormone production during foetal or early postnatal life, severe brain damage can occur. Moreover, even minor changes in thyroid homeostasis and consequent marginally low T_4 levels in pregnant women may give rise to a reduction in cognitive functions of the offspring (Haddow et al., 1999; Pop et al., 2003; Berbel et al, 2009).

Exposure to thyroid-disrupting chemicals (TDCs) may result in a decrease of serum hormone levels and consequent neurological damage. The neurological effects of PCBs have been extensively investigated in humans as well as in animals. Studies in the Faeroe Islands, Germany, the Netherlands, Taiwan and the USA have all reported negative associations between prenatal PCB exposure and measures of cognitive functioning in infancy or childhood (reviewed in Schantz et al., 2003).

It is likely that at least part of the observed effects of PCBs on neurodevelopment is through effects on the function of the thyroid hormone system. Oligodendrocyte development and myelination are under thyroid hormone control, as is the extension of the Purkinje cell dendrites, which is essential for normal neuronal circuit formation (synaptogenesis) and subsequent behavioural functions. Some PCBs and PBDEs as well as BPA have been shown to cause abnormalities in these processes (Kimura-Kuroda et al., 2005; Seiwa et al., 2004).

Immune system

The immune system is a functionally rather than anatomically defined organ, spread throughout the body with a major role of defending the host against infections. It is composed of antigen-specific lymphocytes and their products, constituting the acquired and adaptive component of the host defence, and an innate non-specific part that constitutes the first-line defence. The latter also includes cells that are unable to recognise antigens directly but are nevertheless indispensable to securing a fortunate outcome in the battle against invading microorganisms. Neutrophilic polymorphonuclear leucocytes and cells belonging to the mononuclear phagocyte system (MPS), with monocytes as representatives among blood leucocytes, are important members of this group.

Cells of the immune system are potential targets of EDCs. Numerous substances affect leucocytes and it is difficult to distinguish toxic or immune activating/suppressive effects from those that may count as ED (Ahmed, 2000; Yamashita et al., 2003; Yamashita et al., 2002). Further, there are several reports demonstrating effects of EDCs on cytokine production in various systems (Merlot et al., 2004; Sawai et al., 2003). There are also endocrine effects that indirectly affect immune cells. Thus, EDC-induced changes of immune-modulating hormones such as cortisol may have an impact on the immune system (Merlot et al., 2004). Increased cortisol may hamper the immune defence against pathogens whereas decreased cortisol actions may aggravate autoimmune disorders.

Since immune cells are not typical targets of traditional endocrine hormones, with the exception of glucocorticoids, the relation of such findings to the concept of ED remains to be developed. Examples of EDCs that have been found to exert immune-modulating actions in both in humans and experimental animals are DES, PCDDs, PCBs and PCDFs (Yurino et al., 2004; Calemine et al., 2003; Chalubinski and Kowalski, 2006; Burke et al., 2001; Weisglas-Kuperus et al., 2004; Jung et al., 1998). These effects are mostly weak and the mechanisms are not known in detail. Further, many studies are based on *ex vivo* cultures of lymphocytes

isolated from the research subjects and stimulated by various agents such as polyclonal mitogens, antigen and cytokines. Although significant differences in responsiveness may be registered in such assays *in vitro*, their biological significance *in vivo* is most often unclear.

Adrenal

The adrenal gland is a duplicated organ composed of a central medulla producing catecholamines and an outer cortical layer of cells synthesising steroid hormones. Catecholamines and glucocorticoids (GCs) are involved in acute stress responses of vital importance for survival. The adrenal cortex is under the control of the hypothalamic-pituitary axis linking putative effects of EDCs in the brain to changes in adrenocortical hormone (mainly GC) secretion and action. There is also an important interaction between the adrenal and the immune system, in that GCs are potent immune suppressors. Thus, perturbations of steroidogenesis resulting in changed levels of GCs may indirectly also affect immune functions. Such changes may either be stimulatory or inhibitory on immune responses (*vide supra*). Similarly to androgens and oestrogens, GCs act by binding to nuclear receptors (GR) in cells of target organs.

There are no reports dealing with effects of EDCs on the adrenal medulla, but many studies indicate unwanted actions in the adrenal cortex. There is extensive literature on effects of EDCs and other xenobiotics on adrenal and GR function in experimental animals, and many different factors interfere with glucorticoid homeostasis. DDT metabolites are well-known inhibitors of adrenal function (Lindhe et al., 2001), acting by direct cytotoxicity to adrenocortical cells. Several recent experimental studies show that certain EDCs may adversely affect steroidogenesis in gonads and adrenals *in vivo* and *in vitro* (Whitehead and Rice, 2006). One study in rats (Supornsilchai et al., 2005) demonstrated that the phytoestrogen resveratrol decreased corticosterone production by inhibition of the enzyme 21-hydroxylase (CYP21) after long-term oral exposure via food. Resveratrol is widely used in high doses as a complementary medicine drug, but no data are yet available to support an adverse reaction in humans.

Obesity, metabolic syndrome and pancreatic function

The Western world has experienced a dramatic increase in obesity over the last decades, including increased childhood obesity. Obesity is associated

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with increased risk of many features of the so-called metabolic syndrome, e.g. hyperlipidemia, type II diabetes and high blood pressure. The modern lifestyle, afflicted with overeating and physical inactivity, is most likely a contributing factor, but cannot justify all of the observed increase, nor can genetic factors explain the rapid increase; hence, it has been suggested that environmental factors may be involved. Based on *in vitro* and animal studies, EDCs may be possible candidates (Heindel et al., 2003; Grun and Blumberg, 2006) as fat cell precursors, adipocytes contain oestrogen receptors, and both oestradiol (E2) as well as environmental compounds with oestrogen activity (i.e. BPA) affect the function of pancreatic cells (Alonso-Magdelena et al., 2005; Adachi et al., 2005). However, human studies focusing on the role of EDCs in the aetiology of obesity and/or metabolic syndrome are only now emerging, e.g. the OBELIX project.

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3.2 Wildlife effects

3.2.1 Mammals

Field studies and population-level effects

There are surprisingly few documented examples of ED in wild mammals. It is unclear whether this reflects the fact that ED is rare in wild mammals, or whether it is simply a reflection of how far and few between these studies are. A large numbers of studies with laboratory mammals demonstrate that EDCs can have effects on mammals: cryptorchidism, hypospadias, low levels of testicular hormones, low sperm counts and altered testis pathology in males; and ovarian dysfunction, lowered fertility, endometriosis, leiomyomas, occlusions and stenosis in females. Consequently, there is no reason to think that wild mammals wouldn't suffer ED if they received exposure to EDCs at high enough doses. There must be populations of wild mammals living in contaminated environments that would merit study in this regard.

Only a small number of species of large mammals have been studied. These species have often been predators, which are expected to have the highest body burdens of contaminants, as a consequence of biomagnification. Thus high body burdens of hydrophobic contaminants such as PCBs and brominated flame retardants are found in animals such as seals, porpoises and dolphins, whales, and polar bears (e.g. Aguilar et al., 2002; Hansen et al., 2004; Lie et al., 2004; Ross et al., 2000; Noel et al., 2009; Sonne et al., 2010).

With the exception of the polar bears and the seals, however, there remains minimal evidence that endocrine-disrupting contaminants are having widespread effects on the health of these

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organisms, even though in some animals, the tissue concentrations of particularly persistent organic pollutants (POPs) (e.g. PCBs, PBDEs and OCs) often exceed their general threshold levels of concern. For example, in a recent study on a declining population of killer whales, over 70 % of animals had PCB concentrations that exceeded a 1.3 mg/kg PCB threshold established for ED and immunotoxicity in free-ranging harbour seals (Noel et al., 2009). Moreover, a study of little brown bats, (*Myotis lucifugus*) in the USA revealed the highest concentrations of PBDEs ever found in any wild animal (13 000 ng/g lipid weight); these were thought to be caused by the fact that the bats consume POP-contaminated insects of between 50 % and 100 % of their body weight per day (Kannan et al., 2010).

The reasons for this general lack of evidence are practical: large predators are not common, so few can be studied. It is unethical to kill them for the purposes of scientific research, and no uncontaminated animals are available for comparison. Thus, traditional scientific approaches for ED in top predator mammals are not possible, and so other approaches are required. Often dead or moribund individuals are analysed, or animals killed by hunters are studied. All such approaches come with major obstacles that need to be overcome if exposure to chemicals and ED are to be causally linked. Sometimes 'high profile' mammals receive attention only when they are rare or endangered, by which time too few individuals exist for any conclusive results (Facemire et al., 1995).

Thus there is a conundrum: the species most contaminated with man-made chemicals and hence perhaps the most likely to show evidence of ED, are very difficult, if not impossible, to study in a manner that could convincingly demonstrate causation. Further, because these species contain many different chemicals in their tissues, linking any effects to particular (classes of) chemicals would be very difficult. The many studies aimed at describing and explaining apparently abnormal antler and testicular development in some populations of deer serve to illustrate the problems (e.g. Veeramachaneni et al., 2006). A further example is provided by the Eurasian otter (*Lutra lutra*): it is well known that populations showed a substantial decline across much of its European range during the period from 1960 to approximately a decade ago, when populations started to recover in many areas. Although the dramatic decline in otter populations was generally attributed to contaminants (PCBs, dieldrin and mercury), with the exception of the relationship between PCB exposure, retinoid levels and the health status of the otter (Simpson et al., 2000), there are virtually no biological data to support or reject the role of these three groups of contaminants in otter declines (Christensen et al., 2010)

Yet another complication is that man-made chemicals may affect the health of wild populations, even leading to increased mortality, but the mechanism of action may not be an endocrine one. Probably the most well understood example of the difficulties associated with disentangling endocrine and non-endocrine effects of chemicals is that of the consequences of high POPs concentrations in small marine mammals associated with developmental abnormalities, reproductive impairment, and immunosuppression (e.g. Reijnders, 1986 and De Swart et al., 1994).

Some of these effects are almost certainly due to ED, for example PCB effects on the thyroid axis (Tabuchi et al., 2006; Routti, 2008; Routti et al., 2010). Others, however, such as the association between PCB exposure and immune suppression and/or susceptibility to infectious diseases, although convincing (Troisi et al., 2001; Seibert et al., 2002; Bergman and Olsson, 1985; Bergman et al., 2001) may not be due to an impaired endocrine system (Aguilar and Borrell, 1994; Hall et al., 1992, Beineke 2007; Bernhoft et al., 2000; Lie et al., 2004, 2005).

Polar bears from East Greenland, Svalbard (Norway), and the western Russian Arctic as well as the Baltic grey, ringed and British harbour seals are probably the best studied animals. The estimated daily TEQ for East Greenland polar bears, for example, is between 32- and 281-fold above the WHO sigma TEQ guidelines for humans. Compared to human tolerable daily intake (TDI), these are exceeded for PCB, dieldrin, chlordane and Sigma HCH in East Greenland polar bears (*Ursus maritimus)*. Added to this, the half-lives of these chemicals in polar bears can be 20 years or more.

Many studies report relationships between individual contaminant load and thyroid-related effects in the seal (Brouwer et al., 1989; Hall et al., 2003 and 2007; Routti et al., 2008), sea lions (Debier et al., 2005), beluga whales inhabiting the St Lawrence Estuary (DeGuise et al., 1995), the harbour porpoise (Schnitler et al., 2008) and the polar bear (Braathan et al., 2004; Skaare et al., 2001); this suggests contaminant-mediated disruption of thyroid homeostasis. In some cases, interfollicular fibrosis could be seen in the thyroid gland itself, associated with severe pathological dysfunction in other animals. PDBEs and PCBs particularly

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affect thyroid hormone transport and metabolism (Hallgren et al., 2001; Zhou et al., 2001 and 2002).

Thyroid hormones are described as having a permissive role in the effects of other hormones and various enzymes; they are important for metabolic regulation and are necessary for adequate growth. They control some aspects of fasting and may play a role in moulting cycles (Bentley et al., 1998). They are therefore key components of the endocrine system and any effects on their production, secretion, metabolism and target sites will have consequences for a range of physiological processes.

There are several correlative studies indicating that OHCs may have an impact on reproductive and adrenal hormones in both seals and polar bears (Haave et al., 2003; Oskam et al., 2003 and 2004). In Baltic grey seals, for example, population declines during the 1950s were related to exposure to OHCs (Olsson et al., 1994). Critical to this conclusion was observation of reproductive impairment (Helle et al., 1976a and 1983) and symptoms suggesting immune dysfunction. A high incidence of uterine leiomyomas (benign tumours that depend on ovarian steroids for proliferation) was found to correlate with the body burden of organochlorine contaminants, especially PCBs (Bergman, 1999; Bergman and Olsson, 1989). No prevalence of uterine leiomyomas was reported in grey seals outside the Baltic Sea; in the Baltic ringed seal, leiomyomas were also found but at a much lower incidence.

Some types of PCBs were subsequently shown to have proliferative effects on myometrial cells of the seal *in vitro*, thus suggesting that PCBs may play a part in the growth of the uterine leiomyomas (Backlin et al., 2003a and 2003b). A 60 % decrease in the number of females becoming pregnant occurred in ringed seals from the PCB-contaminated Bothnian Bay. Impacts on recruitment were documented due to decreases in fecundity concurrent with lesions of the female reproductive organs in both types of seal.

More recently, O'Hara and Becker (2003) elaborated on this with a very thorough study of grey seals. Temporal relationships with decreasing OHCs in seals and the reduction of uterine obstructions with a concurrent increase in pregnancies provide additional evidence of a causal link. Numerous other organ systems also appeared to be involved including the adrenal system (adrenocortical hyperplasia and adenomas were found).

It is worth noting that reproductive success among other pinniped species has become a matter of recent concern. Several Alaskan populations of northern fur seal (*Callorhinus ursinus*), the Galapagos sea lion (*Zalophus wollebaeki*) (Alva et al., 2006) and the Steller sea lion (*Eumetopias jubatus*) (Trites et al., 2003) have experienced recent declines, attributed to reduced pupping rate. The causes and timing of reproductive failure are unknown, but in the fur seal, these are suspected to be linked to bioaccumulation of environmental contaminants in maternal body tissue (e.g. Beckmen et al., 2003 and Towell et al., 2006).

Studies of pseudohermaphroditism (abnormal enlarged clitoris) in polar bears and the potential relationship to pollution are not conclusive (Carmichael et al., 2005; Sonne et al., 2006a; Wiig et al., 1998) although a convincing study of the 99 East Greenland polar bears concluded that xenoendocrine pollutants may reduce the size of their sexual organs, likely leading to reduced sperm and egg quality, uterus and penis size and robustness (Sonne et al., 2006b). Reproductive organs from 55 male and 44 female East Greenland polar bears were examined to investigate the potential negative impact from OHCs. Multiple regressions normalising for age showed a significant inverse relationship between OHCs and testis length, baculum length and weight, respectively, and was found in both subadults and adults. Baculum bone mineral densities also decreased with increasing chlordanes, DDT, and HCB in subadults and adults, respectively (all $p < 0.05$). In females, a significant inverse relationship was found between ovary length and total PCB ($p = 0.03$) and total chlordane concentrations CHL ($p < 0.01$), respectively, and between ovary weight and total PBDE ($p < 0.01$) and uterine horn length and HCB ($p = 0.02$). No evidence of uterine tumours, leiomyomas or endometriosis was found in these bears.

In various forms of mammalian wildlife, osteopenia and macroscopic pathology have been examined in bone during distinct periods of exposure to contaminants (Bergman et al., 1992; Lind et al., 2003; Schandorff, 1997; Sonne-Hansen et al., 2002; Zakharov and Yablokov, 1990). The studies showed relationships between organochlorine concentrations and disrupted bone mineral composition. For example, in a study of 139 Greenland polar bears (*U. maritimus*), Sonne et al. (2004) detected that bone mineral density of polar bear skulls sampled in the supposed pre-organochlorine/PBDE period (1892–1932) was significantly higher than that in skulls sampled in the supposed pollution period (1966–2002) for subadult females, subadult males, and adult males (all, $p < 0.05$), but not for adult females ($p = 0.94$).

The strong negative correlative relationships between DDT, total PCB or dieldrin concentrations and bone mineral density suggest that disruption of the bone mineral composition in East Greenland polar bears may have been caused by organochlorine exposure.

The high levels of POPs in Baltic grey and ringed seals has also been associated with deficiencies in bone structure, such as skull lesions (Bergman et al., 1992), and decreased bone density (Lind et al., 2003) and vitamin D3 levels (Routti et al., 2008). Vitamin D3, which is categorised as a hormone rather than as a vitamin, has an essential role in bone mineral homeostasis. A single study to date shows that PCB-exposed rodents had decreased levels of vitamin D3, thus providing a link between PCB exposure, vitamin D homeostasis and bone density. Several studies also indicate that POPs disrupt retinoid homeostasis in free-living seals (Jenssen et al., 2003; Nyman et al., 2003; Mos et al., 2007; Routti et al., 2010). Retinoids (vitamin A and its metabolites) are also dietary hormones and play essential roles in the regulation of growth and development and in the physiological homeostasis of various systems (Novak et al., 2008; Simms and Ross, 2000).

In contrast to the more contaminated Baltic ringed seals sampled a decade ago, more recent studies (2002–2007) on less contaminated seals reported increased concentrations of hepatic retinoids and calcitriol indicating that the health status of these seals has improved in the last decade and adding further strength to the hypothesis linking contaminant exposure to these health effects (Routti et al., 2010).

There are also examples of possible ED in domesticated mammals as a consequence of either natural or conscious contamination of the environment. The best historical case of this was when sheep grazing on clover containing high concentrations of naturally occurring phytoestrogens displayed infertility, dystocia and prolapsed uterus; this was aptly named clover disease (Adams et al., 1995). In addition, female sheep reared on sewage sludge-treated pasture gave birth to lambs with smaller testes than did control lambs (Paul et al., 2005). The authors consider that the most likely explanation for their results is that the mixture of chemical contaminants consumed by the adult sheep caused the reproductive problems in their male offspring.

This may serve as an example of studies that need to be carried out, in that it was possible to compare exposed and control animals. Another example is a study that showed anti-androgenic effects of the fungicide vinclozolin in small rodents feeding on vinclozolin-treated plant material in large enclosures. Although the experiments produced equivocal results (Caslin and Wolff, 1999), the study approach adopted seems a very sound one. Surprisingly, other comparable studies have not been conducted. Controlled field experiments will be required if unequivocal evidence of ED in wild mammals is to be obtained. For field studies, however the biggest scientific challenges revolve around conducting a large enough study to provide robust results, ensuring that the study is representative of the studied environment, and obtaining baseline data from uncontaminated locations. A major ethical problem is the necessity of killing wild animals. Extensive field studies are also very expensive.

Laboratory studies

Even though it is difficult to demonstrate causal relationships in wild mammals, the relationships between the OHC concentrations and reproductive and thyroid dysfunction in seal and polar bear studies conducted over the last decade, strongly suggest that some OHCs alone or as a mixture can modulate the reproductive system in wild mammals. This suggestion is now also supported by results from experimental studies on various model species such as domesticated Arctic foxes, Greenland sledge dogs, and goats (e.g. Lyche et al., 2004a, 2004b and 2004c; Oskam et al., 2004b and 2005; Ropstad et al., 2006; and Sonne et al., 2009a). As a model of high trophic level carnivores, for example, Kirkegaard et al. (2011) exposed female Greenland sledge dogs and their pups to whale blubber contaminated with organohalogen compounds from 2 to 18 months of age, and then examined thyroid hormone status.

Although the sample numbers were low, the results supported observational data in other wildlife and humans, by showing that long-term exposure to OHCs may result in detectable effects on thyroid hormone dynamics by lowering both free and total T_3.

The impact of dietary organochlorine (OC) exposure on thyroid gland pathology has also been studied in farmed male Arctic foxes (*Vulpes lagopus*) (Sonne et al., 2009). The exposed group (n=16) was fed a diet based on wild minke whale (*Balaenoptera acutorostrata*) blubber as a main fat source in order to mimic the exposure to OC cocktails in the Arctic environment. This resulted in an exposure of approximately 17 micrograms Sigma OC/kg day and a Sigma OC residue adipose tissue and liver concentration of 1700 and 4 470 ng/g, respectively, after 16 months of exposure. Control foxes were fed a diet with pork (*Sus scrofa*) fat as a main fat source containing significantly lower OC concentrations. The study showed that the exposed foxes had thyroid gland cysts, C-cell hyperplasia and increased prevalence of cystic remnants of embryonic ducts. Because concentrations of OCs are higher in wild Arctic foxes than in these animals, the authors concluded that it is likely that these animals could suffer from similar OC-induced thyroid gland pathological and functional changes.

3.2.2 Fish

Field studies and population-level effects

At the time of the Weybridge meeting, little was known about ED in wild fish. That situation has changed markedly in the last 10 years, evidenced by a number of robust field studies. The results from these studies demonstrate clearly that ED in wild fish is widespread. The best studied example is the case of the feminised male roach (*Rutilus rutilus*), a cyprinid (carp) fish in rivers throughout the United Kingdom. The prevalence of these male fish with abnormally high plasma vitellogenin concentrations (a female plasma protein), many of which were also intersex (had developing eggs within their testes), was associated with exposure to effluent from STWs (Jobling et al., 1998 and 2006).

Although nonylphenol was initially thought to play a major role in causing this phenomenon, analysis of a nationwide survey suggested that intersex was significantly correlated with predicted concentrations of both natural (oestrone (E1) and 17b-oestradiol (E2)) and synthetic (17a-ethinylestradiol (EE2)) oestrogens, thus suggesting that steroidal oestrogens play a major role in causing ED in STWs effluent–exposed freshwater fish in the United Kingdom (Jobling et al., 2006).

In some locations, however, where the usage and discharge of nonylphenol ethoxylates was particularly high, nonylphenol was evidently playing a major role (Sheahan et al., 2002 and 2009). Once a mathematical relationship was described between a feminising phenomenon in fish and the concentration of sewage effluent in a river, predictive 'effect' maps of the incidence and severity of this phenomenon could be constructed for the whole of the United Kingdom. This concept was first introduced by Jobling (Jobling and Tyler, 2003) and further illustrated by Sumpter, Johnson and Williams with combinations of steroid oestrogens and alkylphenols as the predictors of biological effects (Sumpter et al., 2006). Williams et al. (2009) then constructed the first graphical information system–based model to predict the risk of ED for 10 313 individual river reaches receiving effluent from more than 2 000 sewage treatment plants across the United Kingdom. Overall, 39 % of the modelled reaches were predicted to be not at risk from ED; most of the remaining fish were predicted to be at risk, and a very small proportion (1 % to 3 %) were predicted to be at high risk.

Feminised fish (mainly roach but also chub, bream and gudgeon) have also been found in other European countries such as Denmark, France, Italy, Germany and the Netherlands, and again associated with exposure to STWs effluent (e.g. Bjerregaard et al., 2006a; Vethaak et al., 2005; Hinfray et al., 2010; Viganò et al., 2001; Solé et al., 2003; and Penaz et al., 2005). A Danish study of brown trout (*Salmo trutta*) found elevated vitellogenin concentrations in males from streams impacted with STWs effluent, although no intersex occurred (Bjerregaard et al., 2006b). In North America, initial investigations (e.g. Folmar et al., 2001 and Kavanagh et al., 2004) supported the conclusions of the European studies. A more recent larger scale US study of over 3 000 fish in 9 river basins, intersex was found in only 3 % of the fish collected and in 4 of 16 species examined at 34 of the 111 sites. It was most prevalent in largemouth bass (*Micropterus salmoides*) (18 % of males) and smallmouth bass (*M. Dolomieu*) (33 % of males). The incidence of intersex was greatest in the south-eastern USA, with intersex largemouth bass present at all sites in the Apalachicola, Savannah, and Pee Dee River Basins (Hinck et al., 2009).

ED is also occurring in marine fish. Lye et al. (1997) demonstrated that discharge of STWs effluent to the marine environment caused reproductive

abnormalities in flounders (*Platichthys flesus*). Allen et al. (1999) and Kirby et al. (2004) demonstrated elevated vitellogenin concentrations in many locations, especially estuaries receiving industrial and domestic effluents. Intersex fish were also found, though less frequently.

Similar effects occur in *Pleuronectes yokohamae* captured in Japan (Hashimoto et al., 2000) and in killifish in Newark Bay, USA (Bugel et al., 2010). A comprehensive field survey around coastal Japan using the goby (*Acanthogobius flavimanus*) showed the occurrence of ED near big cities (Ohkubo et al., 2003), albeit with less severity than that found in United Kingdom estuaries.

Even fish in the open sea may be affected by oestrogenic EDCs. Scott et al. (2006) reported that cod (*Gadus morhua*) may be showing signs of ED, larger fish having apparently elevated plasma vitellogenin concentrations, possibly due to bioaccumulation of oestrogenic chemicals via their diet. High vitellogenin levels have also been found in Mediterranean top predator fish such as tuna and swordfish (Fossi et al., 2002; De Metrio et al., 2003), though tuna captured well offshore in the Pacific Ocean did not show any evidence of ED (Hashimoto et al., 2003).

In most studies, oestrogenic effects were documented. Masculinising effects, though apparently less prevalent, have also been reported. In fact, the first evidence of ED in fish was provided by the discovery of masculinised female mosquito fish (*Gambusia affinis holbrooki*) living in a stream that received pulp-mill effluent (Howell et al., 1980); this observation has been confirmed for other countries (e.g. Larsson et al., 2000). Another source of effluent, intensive animal husbandry, has also been shown to masculinise wild fish, due to the presence of anabolic steroids in the effluent (Orlando et al., 2004).

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The consequences of feminisation and masculinisation have been investigated in some fish species. Spottail shiners (*Notropis hudsonius*) in the St Laurence River downstream of the STWs in Montreal had lower sperm counts and lower sperm motility than did fish living upstream (Aravindakshan et al., 2004). Similar effects on sperm production in roach were reported by Jobling et al. (2002a). In this instance, it was possible to show that endocrine-disrupted male roach had reduced fertility (Jobling et al., 2002b).

In addition to the evidence for effects on the reproductive system, there is also evidence in wild fish species for thyroid hormone disruption in contaminated areas. The most famous historical examples were in the salmonids living in heavily polluted regions of the Great Lakes area in the USA (see Leatherland and Sontesgard, 1980 and 1982, reviewed in Jobling and Tyler, 2003). In the last decade, thyroid abnormalities were also reported in mummichogs from a polluted site in New Jersey, USA (Zhou et al., 2000) and in San Francisco Bay, USA (Brar et al., 2010). In the latter study, plasma concentrations of thyroxine (T_4) were significantly reduced in two species of fish from highly contaminated areas, compared with fish from cleaner locations in the same estuary. Both the T_3/T_4 ratio and T_3 concentrations were positively correlated with PCB concentrations measured in the livers of the exposed fish, whilst T_4 concentrations were inversely correlated. Taken together, the results support the conclusions from laboratory experiments and the general hypothesis already indicated in some marine and terrestrial mammals, that environmental PCBs may alter T_4 deiodination or turnover.

Relationships between exposure to other chemicals and thyroid hormone disruption in fish are less common, albeit increasing in the last decade, especially in relation to exposure to the flame retardants (PBDEs). In an interesting laboratory study, Zhang et al. (2009) reported an association between decreased T_3 and T_4 levels and severe damage of the thyroid gland with decreased gonadosomatic index in fish exposed to TBT. This is the first study to suggest that inhibition of thyroidal status induced by TBT might be one of the mechanisms affecting testicular development in fish, as has also been hypothesised in mammals.

There is also evidence to suggest that environmental contaminants can compromise the responsiveness of the hypothalamic-pituitary-interrenal (HPI)

axis. For example, Hontela et al. demonstrated that yellow perch (*Perca flavescens*) and northern pike (*Esox lucius*) from PCB- and PAH-contaminated sites were unable to produce cortisol in response to acute handling stress. Moreover, the adrenocorticotrophic hormone-producing cells (corticotrophs) in these fish were atrophied. The authors speculated that atrophy of these cells was a result of prolonged secretory hyperactivity, a hypothesis later supported by other studies (reviewed in Jobling and Tyler, 2003). Very little work has been carried out in this area since the publication of these results.

In summary, ED is common in wild fish. In many cases, there is a strong association with the presence of effluent, especially from STWs, in the receiving waters. Studies of wild fish — in contrast to laboratory-based tests — have rarely, if ever, convincingly demonstrated population-level effects, even though there is plenty of evidence that some fish stocks are in decline. For example, the status of the European eel, *Anguilla anguilla* is now considered below its safe biological limit, and there is increasing evidence that spawner quality may be an essential element in the decline of this species. Exposure to EDCs could play a role, although extensive research is needed to determine how pollutants are detrimental to eel populations (Geeraerts and Belpaire, 2010).

There is some evidence that other species of wild fish exposed to oestrogenic contamination are likely to be reproductively impaired. In a Canadian study, for example, EE2 was added to an entire lake over three successive years, resulting in ED in fish, including evidence of a reduction in the numbers of young fish in the EE2-treated lake, something that could ultimately affect the sustainability of the population (Palace et al., 2002 and 2006). However, EE2 concentrations in the lake are higher than in rivers receiving STWs effluent, which makes extrapolation of the results difficult.

Laboratory studies

These can be divided into ecotoxicological and analytical studies: the former aim to determine effects of chemicals on fish, whereas the latter aim to identify chemical(s) in the environment responsible for the effects observed. There have been hundreds of studies in both categories in the last 10 years, and hence it is impossible to provide a comprehensive picture.

For example, following on from the demonstration that nonylphenol is weakly oestrogenic to fish (Jobling and Sumpter, 1993; Jobling et al., 1996), well over 100 papers have confirmed this effect. There are a similar number of papers demonstrating feminising effects of E2 and EE2 on male fish at the parts per trillion (ppt) level (e.g. Länge et al., 2001 and Nash et al., 2004). In contrast, all xenoestrogens tested to date were much weaker (e.g. Metcalfe et al., 2001.The effects of the steroid oestrogens have been particularly well studied: there are papers demonstrating effects of these chemicals on sexual behaviour (e.g. Salierno and Kane, 2009, sexual selection (e.g. Saaristo et al., 2009), social dominance hierarchies (Coe et al., 2009) and avoidance of predators, in addition to the many papers on gonadal differentiation and reproduction.

A few laboratory studies have also examined the reversibility of some of the induced effects, such as those on vitellogenin (VTG) production in males and on intersex induction. These studies seem to suggest that feminisation of the germ cells is in some cases reversible when exposed subjects are transferred to clean water, but that the overall fertility of these 'reversed males' never reaches that of the true males, and that courtship behaviour is incomplete. It seems likely that sex-changed, genetically male zebrafish will never develop into fully functional males, despite undergoing major morphological modifications resulting in a male-like morphology (e.g. Larsen et al., 2009).

In addition to oestrogenic chemicals, laboratory tests also encompass those with androgens, anti-androgens, anti-oestrogens and chemicals with the ability to affect steroid hormone biosynthesis. Liu et al. (2010), for example, has shown that the fluorotelomer alcohols (FTOHs) disrupt sex hormone biosynthesis, resulting in impaired reproduction and decreased hatching rates in exposed zebrafish. Although most tests investigated effects of a single chemical, it is general accepted that wild fish are exposed to many EDCs simultaneously. Therefore, it is necessary to know how fish respond to EDC mixtures. A start has been made with studies showing that in mixtures of steroidal oestrogens alone (Thorpe et al., 2003) and mixtures containing steroid oestrogens and xenoestrogens (Brian et al., 2005), chemicals act additively, based on their oestrogenic potencies. Mixtures containing a wider spectrum of EDCs, such as oestrogens and anti-oestrogens, or oestrogens and androgens, have not yet been tested.

Most laboratory tests do not provide any information on long-term consequences of exposure to a chemical. However, exposure tests covering a full life-cycle or even two generations can be performed, if resources allow. Several such studies

with EE2 have been carried out (Länge et al., 2001; Nash et al., 2004; Xu et al., 2008; Soares et al., 2009; Zha et al., 2008b). All of these show that if fish are exposed continuously to low concentrations of EE2, males do not mature sexually, and hence reproduction is prevented. The majority of these studies suggest that if EE2 concentrations in rivers are consistently at or above 1 ng/L, then serious effects on populations can be expected.

An exception is the study of Zha et al. (2008b) in which a complete failure to reproduce was reported in the Chinese rare minnow (*Gobiocypris rarus*) at 0.2 ng EE2/L, which is an order of magnitude lower than that found for the other fish species. Further tests of EE2 in this and other species at across the sub-nanogram per litre range should be conducted to corroborate or refute these results.

To establish exact environmental concentrations of EDCs, a large number of laboratory-based studies have chemically analysed STWs effluent and/or river water. These studies have shown the presence of a large number of EDCs in the aquatic environment. The feminising effects are probably caused by a mixture of natural and synthetic steroid oestrogens and xenoestrogens, and possibly also anti-androgens (Jobling et al., 2009; Hill et al., 2010). In many locations, the steroid oestrogens appear to contribute to most of the oestrogenic activity (e.g. Desbrow et al., 1998), although in some specific locations, xenoestrogens (Sheahan et al., 2002) or phytoestrogens (Kawanishi et al., 2004) can contribute significantly to the total oestrogenicity. Hence it is necessary to conduct site-specific investigations, rather than make assumptions that 'steroid oestrogens are responsible for the feminisation of fish', for instance.

Likewise, the less widely reported masculinisation of female fish probably does not have a single common cause. It is likely that phytosterols are responsible for androgenic activity of some pulp-mill effluents. Natural androgens, originating from people, are present in STWs effluents (Thomas et al., 2002), though effects in fish have not yet been demonstrated. Synthetic androgens have been detected in the aquatic environment: the steroidal growth promoter trenbolone, for example, present in effluent originating from intensive cattle production in the USA (Soto et al., 2004), may cause masculinisation of fish populations downstream of farms (Miller and Ankley, 2004). A variety of non-steroidal man-made chemicals have been shown to possess androgenic and/or anti-androgenic activities. This includes pesticides, phthalates, and UV filters (e.g. Kunz and Fent, 2006 and Gray et al., 1999). However, their contribution to the reported cases of ED of wild fish is unclear.

In summary, hundreds of laboratory studies have been conducted, aimed at determining which chemicals have endocrine activity, what type of activity each has, and which of them actually contributes to the effects observed. What is clear is that many chemicals present in the aquatic environment have endocrine activity. Some are present in very low concentrations but are extremely potent (e.g. natural and synthetic oestrogens and androgens). Others (e.g. xenoestrogens and xenoanti-androgens) are weakly active, and hence much higher concentrations are required to cause effects, but these chemicals can sometimes be present at these concentrations. It seems likely that in many situations, fish are exposed to complex, usually ill-defined mixtures of EDCs, and that it is this mixture, rather than any individual chemical, that causes the effects observed (Brian et al., 2005; Sumpter et al., 2006).

3.2.3 Amphibians

Field studies and population-level effects

Due to their highly permeable integument and the possibility of exposure to waterborne chemicals during critical periods of development, amphibians are potentially vulnerable to EDCs, and in particular to TDCs, because metamorphosis is under thyroid control (Kloas, 2002). Although amphibian populations worldwide are in decline (e.g. Blaustein and Kiesecker, 2002) only a few field studies have examined possible EDC effects on amphibian abundance (Sparling et al., 2001; Crews et al., 2003; Reeder et al., 2005; Davidson and Knapp, 2007). Interest has instead focused on malformations among amphibian populations in the USA, for which a variety of aetiologies have been proposed, with some evidence for a chemical cause (e.g. Ouellet et al., 1997 and Taylor et al., 2005).

Gonadal intersex, feminisation of secondary sexual characteristics and altered sex hormone concentrations have been observed in wild amphibians at sites contaminated by agricultural pesticides across Italy, South Africa and the mid-western USA, parts of Florida, Ontario and Michigan, but exact chemical causality remains uncertain (Bögi et al., 2003; Hayes et al., 2002; Mosconi et al., 2005; Jooste et al., 2005; Eggert, 2004; McCoy et al., 2008; McDaniel et al., 2008).

Considerable debate and controversy surrounds the possible role of the triazine herbicide atrazine in eliciting reproductive abnormalities in frogs (Hayes, 2004), e.g. in cricket (*Acris crepitans*) and leopard (*Rana pipiens*) frogs (Reeder et al., 1998; Hayes et al., 2003). Murphy et al. (2006), for example, found no correlation between agricultural contaminants (including atrazine) at field sites and ovotestes, gonadal steroid levels, aromatase activity and gonadosomatic index in three frog species. Indeed, in their study, testicular oocytes were found in 25 % to 30 % of frogs at both sites categorised as agricultural and those categorised as reference.

Such proportions of testicular oocytes in naturally gonochoristic adults are not likely to be the result of a rare genetic anomaly, since none of the species studied are known to be naturally hermaphroditic. It seems likely, therefore, that the sites classified as reference sites were polluted by some type of endocrine-disrupting contaminant. This highlights another major difficulty encountered when trying to link intersex in fish with contaminant exposure: many sites are designed to compare polluted sites against reference sites, yet there are likely no sites on earth that are not affected by pollution.

Reeder et al. (1998 and 2005) suggested an alternative causality for the temporal and spatial association of intersex in frogs with environmental organochlorine contamination in a retrospective examination of museum collections. The most convincing study, however, is perhaps that by McCoy et al. (2008) in which feminisation of male giant toads (*Bufo marinus*) was associated with agricultural land use in a dose-dependent fashion. No attempt was made by the authors to assign causation to any particular pesticide or herbicide, an unrealistic expectation in a world where multiple types of pesticides and herbicides are distributed globally.

Laboratory studies

Numerous laboratory studies concerning EDC effects on amphibians have been conducted since 1996. Studies cited here do not represent an exhaustive list, but provide an indication of the range of data available.

In bioassay development, *Xenopus laevis* is a well-established amphibian laboratory model, and is the focus of most EDC bioassays. Available ED assays encompass the following: enzyme-linked immunosorbent assays (ELISAs) for the female yolk protein VTG (e.g. Mitsui et al., 2003); hepatic ER binding activity, VTG messenger ribonucleic acid (mRNA) induction in hepatic cell culture, and multivariate assessment of sexual development (e.g. Kloas et al., 1999); recombinant ER binding assay (Huang et al., 2005); hepatic ER transcription assay (Lutz et al., 2005); ex vivo liver slice culture assays for oestrogenic activity (Hurter et al., 2002); *in vitro* germinal vesicle breakdown (GVBD) assays (e.g. Fort et al., 2002); morphological, histological and molecular assays to assess effects on metamorphosis (e.g. Fort et al., 2000 and Opitz et al. (2005, 2006a and 2006b); and an assay to evaluate interference with adrenal steroidogenesis (Goulet and Hontela, 2003).

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For laboratory-based chemical effect studies over the past decade, a wide range of chemicals has been tested but for many, no consensus yet exists on the pattern of effects in amphibians. Most studies have employed *Xenopus* and have focused on chemicals already characterised as having activity in other taxa. In addition, the herbicide atrazine has attracted interest due to its global distribution and high volume production. Atrazine exposure in the laboratory has been reported by some scientists to disrupt sexual development, causing feminisation manifested by abnormal testis size and development, intersex, polygonadism, reduced androgen concentrations and demasculinisation of the larynges of exposed male *Xenopus* (Hayes et al., 2002 and 2006b; Carr et al., 2003), reduced body mass and length at metamorphosis, and increased time to metamorphosis (Sullivan and Spence, 2003).

These effects are suggested to arise at least in part due to the induction of aromatase (Hayes et al., 2006b). However, direct evidence for this mode of action in amphibians is not abundant, not all effects reported have been replicated in several laboratories, and there have been conflicting reports regarding the minimum concentrations of atrazine required to elicit these responses (Coady et al.,

2005; Hecker et al., 2005a and 2005b). The most recent studies by Hayes et al. (2010) and Kloas et al. (2009) muddy these waters even further, as Hayes et al. reports complete demasculinisation and feminisation, reduced breeding success and even sex reversal, in *X. laevis* exposed to 2.5 parts per billion (ppb) atrazine throughout the larval period and for up to three years after metamorphosis. Conversely, Kloas et al. reported that long-term exposure of larval *X. laevis* to atrazine at concentrations ranging from 0.01 μg/L to 100 μg/L did not affect growth, larval development, or sexual differentiation in two replicate experiments conducted in different laboratories. There are many differences between the two studies, not least of which is the fact that Hayes et al. did not publish any measured concentrations of atrazine in their exposure aquaria, whilst Kloas et al. conducted their exposures from 8 days post-fertilisation, compared with the throughout-life exposures conducted by Hayes et al. Kloas et al. performed all of their experiments in flow-through aquaria with a biological loading rate of less than 1 g/L/day, whereas Hayes et al. performed their experiment under semi-static conditions with a 72 hr renewal cycle. Any or all of these factors could affect the comparability of the results of experiments performed.

Orton et al. (2006), for example, showed that atrazine in combination with nitrate (as would be found under greater biological loadings) induced a greater feminising shift in sex ratio among leopard frogs than did either toxicant alone. Moreover, a recent study showed clearly that *X. laevis* collected from north-east sites in South Africa had testicular ovarian follicles, irrespective of exposure to atrazine, while frogs from south-west (SW) Cape region sites had none. Phylogenetic analysis indicates that frogs from the SW Cape are evolutionarily divergent from those from north-east South Africa and the rest of sub-Saharan Africa, and thus provide a possible explanation for why conflicting results have been reported concerning the impact of atrazine on *Xenopus* sexual differentiation (DuPreez et al., 2009).

Many other chemicals have been shown to induce abnormalities in growth or development of the gonad. In laboratory reared tadpoles, for example, glyphosate was shown to induce intersex in premetamorphic tadpoles (Howe et al., 2004) whilst BPA exhibited oestrogenic activity in *Xenopus* tadpoles, inducing significant feminisation and upregulation of ER mRNA (Kloas et al., 1999; Levy et al., 2004). Other compounds reportedly exhibiting ED effects in amphibians include nonylphenol (Mackenzie et al., 2003), DBP (Lee and Veeramachaneni, 2005), DDT and its metabolite DDE (Clark et al., 1998), PCBs (Glennemeier and Denver, 2001; Qin et al., 2003), methoxychlor (Pickford and Morris, 1999 and 2003), triphenyltin (Rehage et al., 2002), the UV screen 4-MBC (Klann et al., 2005), flutamide, vinclozolin and octylphenol (van Wyck et al., 2003).

Perhaps the effects of chemical exposure on metamorphosis in amphibians are of greater interest than are the effects on the gonads. BPA has been shown to block thyroid hormone–induced metamorphosis, indicating anti-thyroid activity (Iwamuro et al., 2003), which is consistent with its antagonism of T_3 binding in *Xenopus* tadpoles (Goto et al., 2006). The herbicide Acetochlor was also found to accelerate T_3-induced metamorphosis of *Xenopus* (Crump et al., 2002b) a process that was preceded by disruption of T_3-dependent expression of thyroid hormone receptor genes in the tadpole tail. Nonylphenol had an overall inhibitory effect on the rate of bullfrog tadpole metamorphosis (Christensen et al., 2005).

A single study has considered the importance of exposure to a composite mixture of contaminants (Hayes et al., 2006a). Nine pesticides were tested singly and in combination. Effects of the mixture exposures were found to be more extensive than predicted on the basis of the single agent results.

3.2.4 Reptiles

Historically, reptiles have received less attention than other taxa in the context of environmental contamination (Campbell and Campbell, 2002). Furthermore, nearly all ED research effort in reptiles has been driven by the progress of studies on alligator populations in Florida. However, laboratory studies since 1996 have addressed an increasing range of species. For reptiles, routes of exposure are more restricted than for truly aquatic animals, with contaminants entering the system either from maternal sources, through the eggshell following oviposition, or via ingested food.

Field studies and population-level effects

The most prominent series of field studies have been carried out in Florida, USA. Lake Apopka suffered an industrial pesticide spill in 1980, resulting in the presence of DDT and its degradation products DDD, DDE and the acaricide dicofol (*p,p'*-dichlorodiphenyl-2,2,2-trichloroethanol), although other potential EDCs are also reported (Semenza et al., 1997). Subsequently, alligator

(*Alligator mississippiensis*) populations declined, stimulating a series of effect studies on the native reptile fauna that have continued since 1996 (e.g. Guillette et al., 2000; Guillette and Iguchi, 2003). Reduced phallus size and altered plasma testosterone concentrations were evident in contaminant-exposed alligator populations (e.g. Crain et al., 1998; Gunderson et al., 2004).

Subsequent studies on other lakes suggested that more diffuse contaminant influx may elicit similar effects (Guillette et al., 1999) including altered hepatic androgen biotransformation activity (Gunderson et al., 2001), spleen and thymus abnormalities (Rooney et al., 2003) and differences in thyroid function (Hewitt et al., 2002). The bone mineral density for pesticide-exposed juvenile females was significantly higher than at uncontaminated sites, suggesting exposure to oestrogens (Lind et al., 2004). The work conducted in Florida highlights the complexities of trying to establish causality where complex contaminant profiles and multivariate effects are concerned.

Few field studies of reptiles outside Florida are available. Male snapping turtles (*Chelydra serpentina serpentina*) from PCB- and PCDD-contaminated sites in southern Ontario (Canada) exhibited feminisation of the precloacal:posterior lobe ratio, although the precise mechanism was not evident (de Solla et al., 1998). Similar observations were reported for hatchling turtles from contaminated sites, suggesting that at least some of the effect arises during early (embryonic) development (de Solla et al., 2002). Shelby and Mendonca (2001) reported minor differences in plasma steroid levels in yellow-blotched map turtles (*Graptemys flavimaculata*) from chemically contaminated and reference sites in Mississippi, USA. These did not appear sufficient to account for the low reproductive rates of animals from the contaminated sites.

Laboratory studies

Reptiles are good models for ED studies because of the range of mechanisms of sex determination they employ (Sarre et al., 2004), and the presence of both oviparous and viviparous modes (Crain and Guillette, 1998).

Extensive laboratory studies have accompanied field investigations of alligators from Florida since 1996. Most contaminants detected in alligator eggs from polluted sites exhibited binding affinity for the alligator ER *in vitro* (Vonier et al., 1996) as did chemicals detected in serum of alligators (Guillette et al., 2002). Toxaphene failed to elicit any developmental abnormalities in alligator embryos, although it has been detected at significant concentrations in eggs from contaminated sites (Milnes et al., 2004). In contrast, exposure of alligator embryos *in ovo* to TCDD (2,3,7,8-tetrachlorodibenzo-para-dioxin) and *p,p'*-DDE did result in a female bias in the sex ratio of hatchlings (Matter et al., 1998; Milnes et al., 2005).

A number of species have been proposed as models, largely based on demonstrating the responsiveness of VTG to exogenous oestrogen (e.g. Brasfield et al., 2002, Selcer et al., 2006 and Sifuentes-Romero et al., 2006). The turtle *Trachemys scripta elegans* has been proposed as a laboratory model (Willingham and Crews, 2000), exploiting the potential for interaction between incubation temperature and hormone environment in determining sex. Trans-and cis-nonachlor, Aroclor 1242, *p,p'*-DDE, and chlordane administered to eggs caused significant feminisation among hatchlings (Willingham and Crews, 1999) but administration of TCDD and PCB-126 to eggs failed to affect sex ratio (Gale et al., 2002), although embryo tissue concentrations reached those reported for environmental samples. Administration of *p,p'*-DDE to snapping turtle eggs at environmentally realistic levels did not induce sex reversal (Portelli et al., 1999), whereas BPA was effective in reversing sex in the broad-snouted caiman (*Caiman latirostris*) at a significantly greater potency than predicted by other non-embryonic assays (Stoker et al., 2003).

3.2.5 Birds

Higher level contaminant effects in birds have been under intensive scrutiny since population declines of the 1960s triggered interest in the role of organochlorines. Initial concerns about effects on mortality via direct toxicity and eggshell thinning have to a large extent receded with the significant reduction in environmental exposure to organochlorines experienced by birds. Sublethal toxic effects on the reproductive axis now assume prominence, although many studies are inconclusive concerning the links between contaminants and ED effects.

Biomarkers of exposure to TDCs have also been evaluated for use with avian subjects by McNabb (2005), Panzica et al. (2005), Viglietti-Panzica et al. (2005) and Grote et al. (2006). Overviews of recent progress (and data pre-1996) in our understanding of ED in birds are available (Dawson, 2000; Ottinger et al., 2001 and 2002; Scanes and McNabb, 2003).

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The mechanisms of sex determination in birds are summarised by Brunström et al. (2003) and these differ significantly from mammals in that the avian female is the heterogametic sex. Prior to differentiation, the avian embryo exhibits bilateral proto-gonadal tissue. In females, the left gonad differentiates into an ovary while the right regresses. In males, both differentiate into testes.

Field studies and population-level effects

Exposure of birds to contaminants is mainly dietary or via maternal transfer to the developing embryo. Top predator birds are particularly vulnerable to contaminants as lipophilic chemicals undergo biomagnification in the food chain. For example, there has been speculation that reproductive and teratogenic effects in a bald eagle (*Haliaeetus leucocephalus*) population from the Great Lakes have arisen from exposure to endocrine-disrupting PCBs (Bowerman et al., 2000). Moreover, tree swallows exposed to PCBs and nonylphenol at a wastewater treatment lagoon exhibited higher levels of PCBs in their blood and lower reproductive performance than did birds from a reference site (Dods et al., 2005).

However, the exact extent to which PCBs exert effects on bird populations is not established, and field studies do not always support extrapolation from laboratory studies (e.g. Fernie et al., 2001, 2003a, 2003b and 2003c), possibly because of differences in susceptibility between species. Ormerod et al. (2000) outlined some of the reasons for this, and provided data from a PCB-exposed dipper (*Cinclus cinclus*) population that indicated no significant effects of exposure. Similar findings were reported for PCB-exposed robin (*Turdus migratorius)* (Henning et al., 2003) and common terns (*Sterna hirundo)* (French et al., 2001) populations.

However, long-term monitoring of herring gulls (*Larus argentatus*) in the Great Lakes revealed significant thyroid dysfunction linked with PCB burden (Scanes and McNabb, 2003), and structural thyroid abnormalities were detected in great cormorants (*Phalacrocorax carbo*) from Tokyo Bay, Japan, effects that were associated with PCDF and PCB contamination (Saita et al., 2004). In addition, negative correlations were found between blood T_4 and T_4:T_3 ratio and levels of organochlorines, particularly hexachlorobenzene and oxychlordane, in glaucous gulls *Larus hyperboreus* from the Barents Sea (Verreault et al., 2003). More recently, high body burdens of PCBs in the European shag (*Phalacrocorax aristotelis*) were associated with increased fluctuating wing asymmetry and also with disruption of the thyroid hormone, vitamin A (retinol) and vitamin E (tocopherol) homeostasis. The authors suggested that this may be due to effects of these compounds on bone growth and bone structure (Jenssen et al., 2010).

Skewed sex ratios and high intersex numbers were detected among juvenile common terns and roseate terns (*Sterna dougallii*) at a PCB-contaminated site in Massachusetts, USA (Hart et al., 2003). However, the ovotestes observed at hatching regressed and did not lead to permanent alterations in gonadal structure. Juvenile female tree swallows (*Tachycineta bicolor*) from PCB-contaminated sites on the Hudson River, USA, exhibited more adult-type colouring than at reference sites (McCarty and Secord, 2000). This was considered of potential functional significance given the importance of plumage in signalling. The colouration is structural rather than pigment based, and is believed to be under hormonal control.

Even though it is difficult to demonstrate absolute causal relationships in wild birds, the relationships between the PCB concentrations and reproductive and thyroid dysfunction in various bird species conducted over a long period, strongly suggest that some PCBs can modulate these systems in wild birds. This suggestion is now also supported by results from experimental studies on various model species.

What is really needed, however, are controlled field experiments in which the diet is consciously contaminated with environmental pollutants, if unequivocal evidence of ED in wild birds is to be obtained. A single recent study, for example, showed that male European starlings (*Sturnus vulgaris*) foraging on invertebrates contaminated with environmentally relevant levels of synthetic and natural oestrogen mimic developed longer and more complex songs compared to control males, a sexually selected trait important in attracting females for reproduction. Moreover,

females preferred the song of males that had higher pollutant exposure, despite the fact that experimentally dosed males showed reduced immune function. This is the first evidence showing that female starlings would bias their choice towards exposed males, with possible consequences at the population level (Markman et al., 2008).

Laboratory studies: The Japanese quail as model species — in ovo exposure routes

The Japanese quail (*Coturnix japonica*) has been widely employed as a model species for EDC effects in birds (Ottinger et al., 2001 and 2005a; Halldin et al., 2005a). It is also a candidate species for an avian two-generation test as part of the Endocrine Disrupter Screening Programme (US EPA) (Touart, 2004). The quail egg has been employed to evaluate effects of EDCs on sex differentiation and gonadal development. The extent of feminisation of male embryos exposed to oestrogens *in ovo* was found to be dose dependent (Berg et al., 1999), with disruption of testes and epididymis structure and function where reversal did not occur (Yoshimura and Kawai, 2002). Embryonic administration of EE2 caused malformations in the reproductive tract (oviduct) of adult female quail (Berg et al., 2001a). Depression of male sexual behaviour (interactions with females) was found to be the most sensitive endpoint in oestrogen-treated males *in ovo* (Halldin et al., 1999). In addition, the progeny of these adults demonstrated reduced fertility (Ottinger et al., 2001).

Administration of weak oestrogens such as *o,p'*-DDT *in ovo* impaired sexual behaviour and reduced plasma testosterone levels in males, while females exhibited inter alia abnormal oviduct structure, changes in shell gland structure and in egg laying (Halldin et al., 2003). Eggshell thinning is a consistently observed consequence of exposure to environmental contaminants in birds. The DDT metabolite *p,p'*-DDE, although not *o,p'*-DDE, *p,p'*-DDT or *o,p'*-DDT, has been shown to interfere with the calcium metabolism of the eggshell gland, an effect that may be mediated by interference with prostaglandin synthesis. Administration of EE2 to quail *in ovo* resulted in a loss of carbonic anhydrase (CA) activity in the shell gland of the adult (Holm et al., 2001), thus adversely affecting eggshell formation. Structural and functional effects on testes and eggshell thinning were also found in domestic fowl exposed to *o,p'*-DDT *in ovo* (Berg et al., 2004; Blomqvist et al., 2006).

Non-steroidal oestrogens have also been studied in the quail. The xenoestrogen methoxychlor exerted pronounced effects on the GnRH system of females treated *in ovo* and depressed male sexual behaviour markedly (Ottinger et al., 2001).

Other studies demonstrated that embryonic exposure to methoxychlor alone (Ottinger et al., 2005b) or in combination with a PCB mixture (Halldin et al., 2005b) affects sexual maturation and male behaviour in quail. BPA injection into eggs induced Müllerian duct malformation in female embryos and testis feminisation in quail and chicken embryos, while TBBPA exerted no oestrogenic effects (Berg et al., 2001b).

There have been few studies that have examined the effects of androgenic and anti-androgenic contaminants on birds. For example, representative androgen-active compounds that have been examined include trenbolone acetate and methyltestostosterone, while vinclozolin has been studied as an example of an anti-androgenic EDC (McGary et al., 2001; Selzsam et al., 2005; Panzica et al., 2007; Quinn et al., 2007a and 2007b).

Laboratory studies: The kestrel and zebra finch as model species — dietary exposure routes

Not all avian studies involving EDCs have utilised *in ovo* exposure. Oral administration of E2 to zebra finches (*Taeniopygia guttata*) resulted in a reduced fertility and increased egg breakage, together with reduced fecundity and hatch rates, while octylphenol elicited no adverse effects (Millam et al., 2001). Orally administered PCBs had significant adverse effects on reproductive indices in American kestrels (*Falco sparverius*) with exposed pairs reproducing later, with smaller clutches and lower fertility, and higher post-hatch mortality than untreated controls (Fernie et al., 2001), as well as with behavioural dysfunction (Fisher et al., 2006) and developmental abnormalities (Fernie et al., 2003c). The results also suggested that non-persistent congeners exerted more effect than persistent congeners.

Oral PCBs did not affect plumage characteristics in the same species but did reduce plasma T_4 levels (Quinn et al., 2002). In contrast, PCB treatment did affect the colour of plumage and plasma carotenoid levels in kestrels given PCBs via the diet (Bortolotti et al., 2003), both endocrine-dependent features. Plasma T_3 concentrations were significantly depressed in PCB-treated kestrels, which also exhibited evidence of immunomodulation (Smits et al., 2002). Intergenerational effects of PCB exposure have also been demonstrated in kestrels, primarily via maternal transfer but also attributable to behavioural effects in the male parent. Where one or both parents

had been exposed *in ovo* to PCBs, the progeny exhibited effects on development and growth, and sexually dimorphic effects on plasma T_3 levels (Fernie et al., 2003b). In zebra finches, orally administered PCB significantly increased numbers of clutches laid and nests constructed per pair, as well as increasing the level of hatchling mortality (Hoogesteijn et al., 2005). Kestrels exposed *in ovo* to PBDEs exhibited a reduced immunocompetence (Fernie et al., 2005).

3.2.6 Invertebrates

Despite the fact that they are evolutionarily distant from the vertebrates, endocrine-signalling mechanisms exist in all invertebrates, although the hormones involved are not always similar to those seen in vertebrates. There is evidence from numerous case studies for chemical effects on development, fecundity and reproduction in invertebrates. In most cases, however, the evidence of an endocrine-disrupting mechanism is rather weak. With the exception of TBT causing imposex in molluscs, and of IGRs as intentionally designed EDCs, there are few field examples of ED in invertebrates. Nevertheless, more cases can be expected. This assumption is supported by the following indications.

1. Endocrine-signalling mechanisms in the animal kingdom exhibit a considerable degree of conservatism (McLachlan, 2001; Keay and Thornton, 2009; Stout et al., 2010). Consequently, at least some features of invertebrate endocrine function would be expected to be affected by similar compounds as those affecting vertebrates (deFur et al., 1999; Pinder and Pottinger, 1999).

2. Highly effective EDCs have been intentionally developed to control insect pests by interfering with their hormonal systems. Their properties are unlikely to be unique and thus it is probable that other chemicals unintended for pest control could also disrupt the endocrine systems of invertebrates.

3. IGRs could also affect other invertebrate groups with similar growth signalling systems to those of molluscs (deFur et al., 1999).

4. ED in invertebrates has received far less attention than it has in vertebrates, probably because their hormonal systems are poorly understood (Oetken et al., 2004).

On the basis of published evidence for ED in invertebrates, the following general observations can be made.

1. Little field work has been carried out on ED in invertebrates, with the exception of TBT and imposex in marine gastropods.

2. Most laboratory studies focus on molluscs, crustaceans and insects, whilst far fewer studies have been carried out on the remaining 27 invertebrate phyla.

3. ED in terrestrial invertebrate species still constitutes less than 10 % of all reports.

4. Marine environments and invertebrates have received little attention, except for imposex in marine gastropods.

Gaps in our knowledge of invertebrate endocrinology are the most important reason for the limited progress made regarding ED in invertebrates in the last 10 to 15 years. Nevertheless, consideration of invertebrates in research potentially offers a wealth of knowledge in understanding comparative and ecological aspects of ED (deFur et al., 1999); this has also been demonstrated in the EU-funded projects 'Comparative research on endocrine disruptors' (COMPRENDO) and 'Identification of endocrine disrupting effects in aquatic organisms' (IDEA).

Therefore, invertebrates should constitute a high priority for further research, especially for the development of EDC screening and testing methods. It will be essential to conduct field monitoring and to perform more basic research on invertebrate endocrinology, especially on thus far neglected systematic groups. Hormone receptors of invertebrates should be recognised, cloned and characterised to identify receptors shared by different groups, opening possibilities for the development of extrapolation techniques for effects between species. This would allow the development of invertebrate *in vitro* systems as screening tools.

The endocrine control of toxicological test endpoints has to be characterised in more depth so that these endpoints can be used as valid measures for ED. New invertebrate tests with endocrine-regulated endpoints have to be developed or existing protocols amended. With few exceptions, our current knowledge on endocrine-mediated endpoints in invertebrates is insufficient to design specific monitoring programmes for EDCs. Nevertheless, carefully targeted monitoring programmes are needed, because effects in invertebrates are probably widespread but still undetected.

a) Endocrine effects and aquatic animals

Along with effects of TBT in prosobranchs and of IGRs in insects, further laboratory and field studies reported effects of compounds on endocrine-regulated processes in invertebrates. For the period until late 1998, the EDIETA report (deFur et al., 1999) summarises not less than 56 studies in which ED may have occurred. While examples for the aquatic environment are almost balanced between freshwater and marine species, only two studies report effects in terrestrial species. Since the publication of the EDIETA report, many new cases of potential ED in invertebrates have been documented.

Porifera

Hill et al. (2002) found a common developmental abnormality and reduced growth rates in the freshwater sponge *Heteromyenia* sp. exposed to ethylbenzene, nonylphenol, and BPA, although an endocrine mechanism was not proved.

Annelida

Depledge and Billinghurst (1999) reported an increased egg production and reduced egg availability in 4-nonylphenol–exposed *Dinophilus gyrociliatus*. Hagger et al. (2002) suggested dose-dependent effects for genotoxic and cytotoxic endpoints in relation to TBT exposure for *Platynereis dumerilii*. Meador and Rice (2001) exposed juveniles of *Armandia brevis* to sediment-associated TBT for 42 days and found reduced growth rates. Bettinetti and Provini (2002) exposed the benthic annelid *Tubifex tubifex* to 4-nonylphenol in the sediment. The production of cocoons and young worms decreased with increasing concentrations of nonylphenol. A histological examination of the clitellum revealed that damage occurred to both male and female gonads. In none of these studies was an endocrine mechanism described.

More recently, however, annelids have been shown to produce and respond to oestrogens. Moreover, functional assays show that an annelid oestrogen receptor specifically activates transcription in response to low oestrogen concentrations and binds oestradiol with high affinity, as in all vertebrate species. Furthermore, numerous known EDCs have been shown to activate or antagonise the annelid ER *in vitro* (Keay and Thornton, 2009).

Mollusca

Molluscan endocrinology, although not so well studied, is of more interest to the study of ED than is insect endocrinology, mainly because there is some evidence to support the existence of vertebrate-type sex steroids and their receptors in this group. Neuropeptides, allied to gonadotrophic neurohormones, oestradiol, progesterone and testosterone, and serotonin as well as prostaglandins have all been reported to be present in some mollusc species (e.g. Tsai et al., 2006 and Croll and Wang, 2007). However, a great deal of work remains to be done to clarify the role of the various hormones in controlling reproduction and development in molluscs as well as their mechanism of action.

Since the EDIETA workshop (deFur et al., 1999), further studies on ED in molluscs have been carried out, most of them in the laboratory using gastropods (for review see Lagadic et al., 2006; Oehlmann et al., 2006a). In field studies, Jobling et al. (2004) compared responses of *P. antipodarum* and fish simultaneously exposed to different dilutions of an oestrogenic effluent. Embryo production of mud snails was significantly enhanced in tanks with 12.5 % and 25 % sewage by a factor of 2, while higher concentrations of the oestrogenic sewage resulted in a decrease (50 % sewage), and even a drop below the control value after 28 and 42 days of exposure (100 % sewage). The same sewage dilutions caused a concentration-dependent vitellogenin induction in male rainbow trout and carp. The concentration response for mud snails exposed to the sewage exhibited the same inverted U-shaped relationship as in laboratory-based studies with oestrogenic chemicals such as BPA, OP and EE2.

Furthermore, reproductive responses to EE2 in the snails were almost identical with those of fathead minnow, with stimulation at low concentrations and inhibitory effects at higher levels. Since these early studies, other researchers have also carried out studies in which snails were exposed to sewage effluents, and/or collected from the field below sewage treatment outfalls (Gagne et al., 2011). They found altered levels of vitellogenin-like proteins in males, feminised sex ratios and low gonadosomatic indices. Moreover, in the estuarine bivalve *Scrobicularia plana,* which is considered to be inherently gonochoristic, varying degrees of intersex were reported in over 20 % of individuals sampled from 17 out of 23 populations in British estuaries (Chesman and Langston, 2006), and in Portugal by Gomes et al. (2009).

Although evidence suggests exposure to contaminants present in sewage discharges increase vitellogenin, egg production and intersex in molluscs, the mechanisms underlying these effects are presently

unclear, despite the fact that many authors refer to these effects as 'oestrogenic'.

Like their sister phylum, the Annelida, molluscs appear to have oestrogen-like receptors but these have not been found to be activated by the vertebrate oestrogen, oestradiol, a range of other steroids, or by known vertebrate endocrine disrupters. *In vitro* studies with ER reporter gene constructs indicate that the mollusc ER is constitutively active when transfected and expressed in mammalian cells.

The most recent studies indicate that chemicals in effluent, including synthetic oestrogens such as EE2, may be operating through pathways controlled by serotonin/dopamine, as both COX and serotonin levels were increased in males with elevated VTG concentrations (Cubero-Leon et al., 2010; Gagne et al., 2011). It is important to note that gametogenesis is not only controlled by steroid oestrogens and androgens, but also by serotonin (5-hydroxytryptamine or 5-HT) and prostaglandins (synthesised by cyclooxygenase (COX). Any or all of these pathways could be disrupted by exposure to EDCs.

Laboratory studies

Despite the lack of demonstration of a mechanism via which oestrogenic chemicals might act in molluscs, Oehlmann et al. (2000) reported 'super females' in the apple snail *Marisa cornuarietis* exposed to the environmental oestrogens BPA and octylphenol (OP). Affected specimens exhibited enlarged accessory sex glands, gross malformations of the pallial oviduct section resulting in an increased female mortality, and a stimulation of oocyte and clutch production.

Enlarged sex glands were also found in BPA- and OP-exposed *Nucella lapillus*, accompanied by a lower percentage of males with ripe sperm, and a reduced penis and prostate length. The results were confirmed by Schulte-Oehlmann et al. (2001), and in two other species of prosobranch snails, *Potamopyrgus antipodarum* and *Nucella lapillus* (Duft et al., 2003).

The first studies on *M. cornuarietis* stimulated a great deal of argument and have potential political importance; they indicate that BPA may cause effects at doses that are ecologically relevant and that are lower than those reported to cause effects in all other aquatic animals tested. Indeed, the controversy surrounding the validity of these studies led Oehlmann et al. to repeat their own studies (Oehlmann et al., 2006) and to again demonstrate a superfeminisation syndrome in *M. cornuarietis.*

Oehlmann et al. (2006) concluded that BPA acts as an ER agonist in *Marisa* because effects were completely antagonised by a co-exposure to the ER antagonists tamoxifen and ICI 182780. These studies also demonstrated that both spawning time and temperature influence BPA disruption of snail reproduction. Before and after the spawning season, super female responses were observed which had been absent during the spawning season. The adverse effect of BPA was at least partially masked at 27 ˚C when compared with 20 ˚C.

In complete contrast to these studies, other research found that 12-week exposure to 0 mg/L, 0.1 mg/L, 1.0 mg/L, 16 mg/L, 160 mg/L or 640 mg/L BPA had no effect on reproduction, egg hatchability, or timing of egg hatching in ramshorn snails (Forbes et al., 2007 and 2008).These results do not appear to support those of Oehlmann, although there were significant experimental design differences between the Forbes study and those of Oehlmann, as well as differences in the behaviour of the snails themselves. For example, the snails used in the Forbes study did not have a seasonal reproductive cycle and were kept in pairs rather than in groups.

Seasonal differences in the response of molluscs to exposure to oestrogens/xenoestrogens were also seen in *Mytilus edulis* exposed to E2 (Ciocan et al., 2010). In Sydney, Australia, rock oysters were exposed to a range of concentrations of 17 alpha-ethynylestradiol (EE2) (0 ng/l, 6.25 ng/l, 12.5 ng/l, 25 ng/l or 50 ng/l), suggesting oestrogenic exposure is capable of facilitating a progression of protandrousmales from male to intersex to female status, at the commencement of a gonadal development cycle (Andrew et al., 2010). Again, the mechanism(s) underlying these responses is not known.

It has been shown that potent AR agonists and aromatase inhibitors as well as the TBT induce imposex in prosobranch female snails, a condition in which the penis 'imposes' on the normal female reproductive anatomy. The associated development of the vas deferens can in extreme cases lead to blockage of the oviduct of the female, resulting in sterility. The effects of TBT are the best known example of ED in invertebrates, and have been reported in a number of gastropod molluscs.

No other types of chemicals were found to induce this response until Schulte-Oehlmann et al. (2004) and Albanis et al. (2006) demonstrated that the androgen methyl testosterone (MT) could also induce imposex and cause inhibitory effects on spermatogenesis and egg production. Moreover, Tillmann (2004) exposed the netted whelk *Nassarius*

reticulatus to fenarimol (FEN) via sediments and found a time- and concentration-dependent increase of imposex. These results were confirmed by Albanis et al. (2006) for *M. cornuarietis*.

Tillmann et al. (2001) communicated a suppressed imposex development in snails co-exposed to TBT and either cyproterone acetate (CPA) or vinclozolin (VZ); both of these are anti-androgens. If administered alone, both anti-androgens reduced the length of male sex organs (e.g. penis and prostate). For *Marisa*, this effect occurred only in sexually immature specimens and was reversible as the males attained puberty.

Since testosterone itself appeared to induce imposex in some species, it was suggested by various authors (Spooner et al., 1991; Bettin et al., 1996; Santos et al., 2002) that TBT and testosterone may competitively inhibit P450 aromatase activity, thereby preventing the conversion of androgens to oestrogens (and consequently increasing testosterone levels), or inhibiting testosterone excretion or decreasing the esterification of testosterone (Gooding et al., 2003). Despite these observations, attempts to isolate an AR from molluscs have been unsuccessful and so the mechanism through which these apparent 'androgenic' effects are occurring is unknown and controversial (Sternberg et al., 2008a).

The only convincing body of evidence for mechanisms of masculinisation in molluscs indicates that tributyltin-induced imposex involves the abnormal modulation of the retinoid-X receptor (RXR) (Kanayama et al., 2005; Nishikawa et al., 2004; Castro et al., 2007; Lima et al., 2011), also known to be involved in male reproductive differentiation and external genitalia formation in mice (Kastner et al., 1996; Ogino et al., 2001). The involvement of steroid hormones and/or their receptors in this process is unknown.

Crustacea

Crustaceans are likely to be highly vulnerable to diverse chemicals in the environment affecting their endocrine systems. As in molluscs, however, little is known about the endocrine systems of the Crustacea. Control of reproductive development, for example, requires neuropeptides, ecdysone and methyl farnesoate (Nagaraju et al., 2011) but little is known about the identities of environmental contaminants that may disrupt these signalling processes at environmentally relevant concentrations.

Studies are required in the field of neurohormone (serotonin and dopamine) regulation of reproduction and in moulting-related parameters (regulated by ecdysteroid hormones). Moreover, some sex steroid hormones, known from vertebrates (testosterone and progesterone) have been found in Crustacea, but knowledge about their targets and signalling is still limited (Mazurova et al., 2008).

DeFur et al. (1999) summarised a large body of EDC effects in crustaceans, which was updated relatively recently by LeBlanc (2006). For example, the similarity of the crustacean hormones to the juvenile and ecdysteroid hormones of insects makes them vulnerable to the effects of juvenile hormone mimics as well as ecdysteroids used to control insect pests. There are no confirmed examples of this having occurred in the wild, although population declines in the American lobster (*Homarus americanus*) on the eastern seaboard of the USA were hypothesised (but not proved) to be caused by the widespread prophylactic application of the juvenile hormone mimic methoprene across New York City and Connecticut to control mosquitoes (Biggers and Laufer, 2004; Walker et al., 2005).

Further examples of ED in wild species of Crustacea undoubtedly exist as numerous laboratory studies (e.g. Kenney and Celestial, 1993; Arnold et al., 2009; McKenney, 2005 and LeBlanc, 2007) have shown that juvenile hormone agonist mimics inhibit metamorphosis and reproduction in crustaceans. Moreover, in *Daphnia magna*, neonatal exposure to the juvenile hormone analogue Piroxifen or methyl farnesoate has been shown to reduce vitellogenesis (Tokishita et al., 2006), cause intersex (Olmstead and LeBlanc, 2007), and skew the sex ratio towards males (Baskay et al., 2007).

Exposure to vertebrate steroid hormones such as oestradiol and testosterone has also been shown to inhibit reproduction in *D. magna* and in other Crustacea, albeit at extremely high concentrations not thought to be physiologically or environmentally relevant (e.g. Vogt, 2007). Similarly, in amphipods (mainly *Gammarus* spp.), EE2 and BPA as well as oestrogenic sewage effluent can disrupt reproductive behaviour male/female body size differential and gonad development at comparatively high concentrations, where it would be unrealistic to attribute the effects to an endocrine-mediated process (Watts et al., 2001b; Gross-Sorokin et al., 2003; Gross et al., 2001).

Data on effects of chemicals known to cause ED in vertebrates in other crustacean groups are also available. Brown et al. (1999) exposed *Corophium volutator* to nonylphenol (NP), an oestrogenic chemical in vertebrates known to bind to and

activate oestrogen receptors. At 10 μg/L, the density of surviving specimens was reduced, growth was retarded and female fertility increased. Sex ratio was not affected; however, the second antennae of exposed male animals were significantly longer than those of control animals, restricting their ability to move and posing a selective disadvantage.

The mechanism underlying these effects is unlikely to be an oestrogenic mechanism. Billinghurst et al. (2000) examined the effects of exposure to 4-NP and E2 on *Balanus amphitrite* and found elevated levels of the cypris major protein (CMP), which is related to barnacle yolk protein, vitellin. Oberdörster et al. (2000) observed a significant increase of vitellin in female grass shrimps (*Palaemonetes pugio*) at 63 μg/L of the PAH pyrene.

In general, chemicals known to affect steroid hormone-regulated processes in vertebrates can also affect comparable endpoints in crustaceans, but the mechanisms underlying these effects are unclear. Further research of crustacean physiology and comparative studies with various EDCs will help to understand mechanisms of action as well as ecological risks of EDCs in the environment for these animals.

Insecta

Aquatic insects play an important ecological role, especially in freshwater environments. Insect hormones, including the peptide hormones, ecdysteroids and juvenile hormones regulate moulting and metamorphosis, yolk synthesis, diuresis, mobilisation of fuel for flight, polyphenism (occurrence of several phenotypes in a population that are not attributable to genetic differences) and diapauses.

ED has been studied very little in insects, except in relation to the IGRs, a group of insecticides developed to intentionally mimic, block or otherwise interact with the hormonal system of insects. DeFur et al. (1999) summarised 47 references for IGR studies on non-target species. Occasionally, chitin synthesis inhibitors (CSIs) such as Diflubenzuron (Ishaaya, 1992) have been misreported as EDCs. Although they interfere with moulting as an endocrine-regulated process in arthropods, the mechanism of CSI action is non-endocrine. CSIs cause death during the moult, resembling the same effect as endocrine mimicking IGRs, albeit not via an endocrine pathway (blocking chitin synthesis). Since the publication of deFur's book, additional evidence for ED in insects has been presented, mainly related to the disruption of the ecdysteroid and juvenile hormone systems.

Insects cannot synthesise steroids, but use sterols obtained through the diet to produce ecdysone, a prohormone that is converted to 20-hydroxyecdysone (20E) in the fat body or epidermis by a cytochrome P450 enzyme. In the honeybee *Apis mellifera* (Hymenoptera) and in the Heteroptera, the principal ecdysteroid is makisterone that exerts its effects through a heterodimer protein composed of the ecdysteroid receptor and ultraspiracle (USP), the insect ortholog of the RXR (Yao et al., 1993). The receptor complex then binds to an ecdysone response element within the promoters of target genes, altering their expression and leading to changes in genetic programmes associated with moulting and metamorphosis.

In contrast to the ecdysteroids, much less is known about juvenile hormones and their receptors in insects (Dubrovsky et al., 2005). The major roles of these hormones are to modulate ecdysteroid action and to act as gonadotrophs, stimulating vitellogenesis, inducing gonad growth and maturation.

There are various reports of chemically mediated ED caused by insecticides affecting hormone signalling in non-target aquatic invertebrates. Hahn et al. (2001), for example, exposed the midge *Chironomus riparius* to Tebufenozide, an ecdysone mimic used to control Lepidoptera pests in fruits and vegetables and other crops. The exposure caused pupal mortality, with long-term exposures causing effects at concentrations 20-to-30–fold lower than those at which acute effects were observed. Moreover, pupal mortality was twice as high in males as in females, but the mechanism underlying this effect is not known.

DeLoof and Huybrechts (1998) have suggested that ecdysone might act both as a direct precursor of 20E and as a male sex hormone. Accordingly, Tebufenozide might stimulate a higher 20E and thus lower ecdysone environment when it binds to the ecdysone receptor. Moreover, the induction of enzymes that metabolise ecdysone into 20E may lead to an even bigger decrease in ecdysone in the males, resulting in greater effects of this pesticide on males than on females. Hahn and Schulz (2002) reported sex-specific effects of TBT on moulting-hormone biosynthesis and imaginal-disc development in *C. riparius* with a decreased ecdysteroid synthesis in female larvae and an increase in males. The use of vitellogenesis as a marker for possible effects of EDCs was

tested by Hahn et al. (2002). The authors found an alteration of vitellogenin/vitellin production in BPA- and NP-exposed males. In a two-generation experiment with *C. riparius*, Watts et al. (2001a) demonstrated that median emergence times (EmT50) and the percentage of adult emergence were affected by EE2 and BPA exposure. These effects were primarily associated with the second generation of test animals, most notably in the BPA study, where the emergence of male and female adults was significantly delayed at concentrations ranging from 78 ng/L to 750 μg/L. At very low concentrations (1 ng/L) of EE2, both the first and second generation of adults emerged significantly earlier than did control animals.

As with the Crustacea, with the exception of the IGRs, the mechanisms of action of the various vertebrate EDCs on the endocrine systems of insects are not known.

Rotifera

The discovery of oestrogen receptor homologues in molluscs and annelids suggests that lophotrochozoan lineages have not lost this type of hormone chemoreception, despite the use of non-androgen steroid hormones in ecdysozoans (e.g. ecdysteroids in insects, and dafachronic acids in *Caenorhabditis elegans*). Very recently, Stout et al. (2010) provided convincing evidence for the existence of a progesterone receptor in the invertebrate monogonont rotifer *Brachionus manjavacas* as well as evidence for a role in sexual reproduction. Previously, Preston et al. (2000) had analysed reproductive effects of potential EDCs in the freshwater rotifer *Brachionus calyciflorus*. Flutamide, testosterone, and NP inhibited fertilisation of sexual females, while asexual reproduction was not affected, pointing at an endocrine mechanism, although not confirmed at that time. In a further study using *B. calyciflorus*, Radix et al. (2002) found a decrease in the total number of females following exposure to EE2, NP and testosterone.

Echinodermata

DeFur et al. (1999) cite seven studies using echinoderms, mainly the starfish *Asterias rubens* and testing effects of heavy metals, PCBs, PCP and E2. Echinoderm regeneration following autonomy is an important asexual mechanism of reproduction and is largely under endocrine control. Therefore, it provides a tractable test to monitor the effects of EDCs in echinoderms (Carnevali et al., 2001a and 2000b). The crinoid *Antedon mediterranea* is especially sensitive to PCBs. Exposure to Aroclor 1260 results in abnormal arm growth (Carnevali et al., 2001a) with hypertrophic development of coelomic canals and compromised tissue differentiation.

b) Endocrine effects in terrestrial invertebrates

Compared with the existing evidence for ED in aquatic invertebrates, possible effects of EDCs on terrestrial invertebrates have been given far less attention.

Earthworms were used to assess EDC effects on annelids. Ricketts et al. (2003) analysed vitellogenin production in *Eisenia fetida* in response to environmental oestrogen exposure. Widarto et al. (2003) report effects of NP on population growth rate of *Dendrobaena octaedra*, although without any proof for an endocrine mode of action.

Dinan et al. (2001) developed a cell line from *Drosophila melanogaster,* the B-II bioassay, as a screening tool for ecdysteroid receptor agonists and antagonists. About 80 environmental contaminants, including industrial chemicals, pesticides, pharmaceuticals, phytoestrogens, and vertebrate steroids, were compared with data for known (ant-) agonists. Apart from androst-4-ene-3, 17-dione and EE2, vertebrate steroids were inactive at concentrations up to 10^{-3} M. The vast majority of xenobiotics did not exhibit any activity, excepting BPA, diethyl phthalate and some organochlorines (DDE, dieldrin and lindane).

Dedos et al. (2002a and 2002b) studied the relationship between Fenoxycarb levels and general esterase (GE) activity, juvenile hormone esterase (JHE) activity, and induction of permanent fifth instar (dauer) larvae in the silkworm *Bombyx mori*. Fenoxycarb treatment induced dauer larvae with persistently increased haemolymph GE and JHE activities for the rest of the fifth instar.

Conclusions

To date, most investigation of invertebrate endocrinology, and thus understanding of endocrine-disrupting effects of chemicals, have focused on insects. Perhaps paradoxically, however, most reported effects due to potential ED have been reported in taxa about whose endocrinology we know very little. Even within relatively well-researched areas, there are enormous gaps in our knowledge. Further research in this area is needed, especially bearing in mind the dependence of many vertebrate food chains on invertebrate biodiversity and abundance.

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3.3 Mechanisms and laboratory animal models

3.3.1 Overview of current in vivo animal and in vitro research

Concern about EDC effects on humans and wildlife arose in 1992 following publication of the proceedings and the consensus statement from a meeting sponsored by the the World Wide Fund for Nature (WWF) and organised by Dr Theo Colborn. The meeting consensus acknowledged that chemicals that display endocrine activity could have profound effects on reproduction of wildlife and humans, especially during development. EDC-induced alterations were clearly evident in wildlife and plausible in humans. The effects of the insult could be latent for decades and serious in nature, and many effects would not be detected in standard toxicological testing. EDCs could act by mimicking natural hormones, by altering hormone synthesis or by interfering with hormone signalling pathways.

In response to these concerns, the EU and USA held meetings in 1996 to address the concerns about EDCs, identify data gaps, and propose research to fill these data gaps. The US EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), and the European Commission held the Weybridge workshop. This discussion will review the questions posed at the Weybridge workshop, the research needs, and the research accomplishments over the last 15 years that have addressed these needs. Were the right questions asked? Have the questions been answered or not? What new questions have arisen and what scientific innovations have taken place in this time frame that enhance our understanding of the issues?

Study of the effects of EDCs involves the use of *in vitro* and *in vivo* assays. In the last 15 years, new *in vitro* assays for EDCs have been developed that enhance our ability to study mechanisms of action of these chemicals without using animals. Assays for interactions of chemicals with recombinant hormone receptors from multiple vertebrate species can now be studied and compared *in vitro* (e.g. Freyberger et al., 2010; Freyberger and Ahr, 2004; Kim et al., 2010; and Witters et al., 2010), whereas the assays proposed 15 years ago primarily relied upon animal tissue derived receptors. In addition, there are several new assays that allow screening of large numbers of chemicals *in vitro* for hormone activity in order to discriminate hormone agonists from antagonists. Similarly, *in vitro* assays are being validated that can assess the effects of chemicals on hormone synthesis without using animal derived tissues, as proposed in 1996.

Computational models (including structure–activity relationship (SAR), quantitative SAR (QSAR), three dimensional QSAR (3D-QSAR) and comparative molecular field analysis (CoMFA) models of EDC action have been developed using the data generated in these assays (e.g. Vedani et al., 2009), and it remains to be seen how useful they will be in prioritising chemicals for screening and testing. In all cases, the aim is to predict receptor binding or activity from the properties of the ligand. These assays also allow comparison of the effects of different chemicals on receptors of different vertebrate classes. Such data can help answer questions posed: whether effects observed in fish are relevant to humans, or if a screening battery based only upon mammalian assays may be predictive of effects in lower vertebrates, for example.

There are several relevant databases for EDC effects. The Endocrine Disruptor Knowledge Base (EDKB) contains data from more than 1 800 putative endocrine disrupters, and ToxCast (Reif et al., 2010) contains 300 well-characterised chemicals that were profiled in over 500 high-throughput *in vitro* screens. There is also Tox21 (Shukla et al., 2010). All of these use *in vitro* methods to prioritise compounds for further study, identify mechanisms of action and develop predictive models for adverse health effects.

To date, the ultimate determination that a chemical is an endocrine disrupter relies upon identification of effects *in vivo*. Only *in vivo* can

absorption, distribution, metabolism and excretion of a chemical be accounted for. A major part of the *in vivo* research on EDCs in the past decade has focused on male reproductive effects of, for instance phthalates, oestrogens, plastic additives, pesticides, dioxins and PCBs, and knowledge on the effects and modes of action of, for instance, phthalates, in animal experiments has increased considerably.

In the last 15 years, epidemiological studies have refined the hypothesis regarding adverse trends in human health, resulting in new laboratory research questions to be answered in order to determine the biological plausibility of the possible link between human exposures and effects. However, while there have been significant advances in understanding basic endocrinology and effects of EDCs on these processes, many questions remain about the effects of EDCs on pubertal development, obesity, some human diseases (cancers), and reproductive health trends.

Advances in new technologies such as genomics and proteomics enable the study of molecular pathways of EDC action not only *in vitro*, but also *in vivo*. The wide availability of transgenic mouse models for specific genes has led to the discovery of new modes of EDC action and enabled scientists to study molecular pathways *in situ* in much greater detail. Scientists are now also working to link human and laboratory exposures to EDCs by measuring the active metabolites of EDCs in human and animal tissues at the time of development of the lesion. Such data are expected to lead to reduced uncertainty in extrapolating effects from *in vitro* and *in vivo* laboratory studies to humans or wildlife, since exposure levels in the laboratory can then be compared with environmentally relevant exposure levels.

3.3.2 Mechanisms of endocrine disruption

There are limited ways in which EDCs can act, these being determined by the manner in which endogenous hormones act. Hormones must be synthesised and secreted, then delivered to their sites of action, classically via transport in the bloodstream. They then interact with specific receptors, which subsequently initiate downstream signalling processes that result in biological effects. A further, and largely overlooked, event is that the hormone must be metabolised if its actions are to be delimited (i.e. regulated). Each of these steps in hormone production and action therefore offer potential points at which environmental chemicals could act and modify (induce, block or exacerbate) hormone action.

A decade ago, the focus of both concern and action regarding EDCs was on hormone receptor agonists and antagonists, in particular ER agonists. As a consequence, most research effort at the time was directed at establishing *in vitro* and *in vivo* systems for the detection of ER-mediated oestrogenic activity in chemicals. Thus, Michigan Cancer Foundation-7 (MCF7) breast cancer cells or other cell lines transfected with ER constructs were used to screen large numbers of chemicals for oestrogenic activity. Some of these positive chemicals were then tested *in vivo* in the rat uterotrophic assay, which was considered more predictive of whether or not the test chemical might be capable of inducing oestrogenic effects *in vivo*.

In both *in vitro* and *in vivo* oestrogenic assays considerable effort was made to establish the oestrogenic potencies of test chemicals relative to E2, as the more potent chemicals were considered of potentially more concern. Similar *in vitro* and *in vivo* (Hershberger assay) assays were established for screening chemicals for androgenic and anti-androgenic activity. These screening and evaluation systems for ER- or AR-mediated hormone activity continue to be widely used.

However, in the past decade, the scope of EDCs has expanded considerably. There is more concern about EDCs that affect other hormone systems (e.g. thyroid), EDCs with new modes of action (e.g. inhibitors of endogenous hormone production or metabolism), and target tissues for EDCs other than those in the reproductive system (e.g. brain, metabolic system and cardiovascular system). There is also additional new evidence that some common environmental chemicals have the potential to exert multiple hormone-modifying effects (e.g. PCBs).

Overlaying these developments has been the evolving saga of the so-called low-dose EDC issue, which has sharply divided scientific opinion and which raises, amongst other issues, the (confusing) potential for non-hormone, as well as hormone, effects of certain EDCs on hormone-sensitive tissues.

Perhaps the most important new (and most recent) mechanistic development relates to the complex issue of the effects of mixtures of EDCs. As is discussed below, new *in vitro* and *in vivo* studies have now shown that chemicals present individually in a mixture at concentrations that are without

significant effect can together as a mixture induce biologically meaningful effects.

Such findings have opened the door on the more difficult issue of how to assess the effects of chemical mixtures in which the components have different EDC activities and/or different modes/mechanisms of action. These are likely to figure highly in EDC research over the next decade, and have important implications for safety evaluation and regulation of EDCs.

Finally, the past decade has seen a considerable expansion of understanding of health disorders in humans and wildlife that have ED as a central feature, thus raising the possibility (still largely unproven) that EDCs could contribute aetiologically to these disorders.

Reproductive-related disorders continue to dominate this area, but neurodevelopmental and thyroid hormone–mediated disorders have also joined the list. With the increasing prevalence of lifestyle-related disorders (e.g. obesity, type II diabetes) in which disruption of endogenous hormones plays a central role, a major task for the next decade will be to distinguish these effects from the potential effects of EDCs.

3.3.3 Mixture effects of EDCs

Both the Weybridge workshop and the EDSTAC final reports (1998) recognised the need for research on how mixtures of EDCs act. Humans and wildlife are rarely, if ever, exposed to a single chemical at a time. At the time, EDC mixture research was in its infancy, but in recent years it has become an active research area and much has been learned (reviewed in Kortenkamp, 2007 and 2008). While most of the initial research was conducted *in vitro*, more research is now being conducted *in vivo*, and a few studies include developing animals.

The research field on mixture effects of EDCs has been one in which progress has been made in direct response to the needs of the regulatory agencies. In the late 1990s, discussions arose on potential synergistic effects of oestrogenic chemicals; these discussions also stimulated research and drove developments within this field. The *in vitro* evidence at the moment points to the fact that synergism between EDCs is not a common phenomenon. Most *in vitro* and *in vivo* studies involving ER- and AR-mediated responses and developmental toxicity studies on similarly acting EDCs have come to the conclusion that EDCs frequently act in an additive fashion. This means that exposure to mixtures composed of low levels of several EDCs, which on their own do not show any effect, often may result in marked effects together; these effects are appropriately termed dose additive (and not synergistic).

The research on mixture effects of EDCs focused initially on oestrogenic effects *in vitro*. In most cases, results have shown additivity for xenoestrogens (Payne et al., 2000 and 2001; Rajapakse et al., 2001 and 2002). Mixture effects have been further analysed for compounds that were present in mixtures at concentration levels below their individual no-observed-effect concentration. Mixture effects were first demonstrated with 4 compounds (Payne et al., 2001) and later confirmed with mixtures of 8 and 11 xenoestrogens (Silva et al., 2002; Rajapakse et al., 2002).

Thus, even though each compound does not individually have any effect, the total burden may have an integrated effect, showing that effects of 0+0 can actually be 2 without necessarily invoking synergism. This phenomenon, somewhat provocatively dubbed 'something from nothing' (Silva et al., 2002), has been observed with multicomponent mixtures of oestrogenic agents in reporter-based assays (Rajapakse et al., 2002; Silva et al., 2002), the uterotrophic assay (Tinwell and Ashby, 2004) and vitellogenin induction in fish (Brian et al., 2005). 'Something from nothing' effects should occur even when individual toxicants are present at doses below effect thresholds, provided sufficiently large numbers of components sum up to a suitably high total effect dose.

A relatively limited number of studies on mixture effects of anti-androgenic EDCs have been published so far. Anti-androgenic EDCs that act by blocking ARs have been found to act additively *in vitro* and *in vivo* in Hershberger assays (Birkhoj et al., 2004; Gray et al., 2001; Nellemann et al., 2003; Price et al., 2000). Additivity was shown irrespective of the molecular complexity of the endpoint measured, i.e. at organ weight level, hormone level and gene expression level (Nellemann et al., 2003).

Little is known about the developmental effects of *in utero* and early postnatal exposure to multiple EDCs. A few studies have recently examined mixtures of anti-androgenic chemicals that disrupt male rat reproductive tract development via a common receptor-mediated mechanism, i.e. AR antagonists. In one study, mixtures of three AR antagonists were investigated for their ability to induce disruption of male sexual differentiation after *in utero* and postnatal

exposure (Hass et al., 2007). Using anogenital distance as the endpoint, the joint effects of the two or three anti-androgens were essentially dose additive. In newborn male pups from the same study, mixture effects on androgen-mediated gene expression in ventral prostates was investigated using the above-mentioned approach, and the same conclusion was drawn, namely that additive effects also occur at the molecular level (Metzdorff et al., 2007).

Administration of mixtures of phthalate esters that disrupt sexual development *in utero* via a common mechanism of action (reducing foetal testis hormone synthesis) also appear to disrupt male rat reproductive tract development in a dose-additive manner (Gray et al., 2001 and 2005; Howdeshell et al., 2008). Thus, so far, the dose addition approach seems to be able to provide a solid basis for prediction of joint effects of multicomponent mixtures of receptor-mediated anti-androgenic action.

Mixture studies are now also considering how EDCs that disrupt common developmental pathways via diverse modes of action will interact. A recent study on a more complex mixture of seven chemicals (four pesticides and three phthalates) indicated effects that were dose rather than response additive (Gray et al., 2005; Christiansen et al., 2008; Christiansen et al. 2009; Rider et al., 2009). Unusually, Christiansen (2009) reported synergisms with respect to the occurrence of penile malformations in the offspring of rats that had been dosed with a mixture of phthalate DEHP, two fungicides, vinclozolin and prochloraz, and a pharmaceutical finasteride, although the mechanism for the synergism was not found.

ED from environmental contaminants has been linked to a broad spectrum of adverse outcomes, including effects on thyroid homeostasis. A short-term animal model to examine the effects of environmental mixtures on thyroid homeostasis was developed (Crofton et al., 2004). Prototypic TDCs such as dioxins, PCBs, and PBDEs were shown to alter thyroid hormone homeostasis in this model, primarily by upregulating hepatic catabolism of thyroid hormones via at least two mechanisms. There was no deviation from additivity at the lowest doses of the mixture, but at high doses the additivity model under-predicted the empirical effects by two- to three-fold, i.e. these TDCs seemed to act synergistically at high doses. Further studies are needed in order to evaluate the significance of these findings on TDCs.

Currently, several laboratories in the EU and USA are beginning to study how chemicals from different classes that act via divergent mechanisms of action interact when administered to rats *in utero* or during puberty. This could have a major impact on the regulatory community, since either the effects of mixtures have been ignored in current risk assessments or focus has been placed only on mixtures of chemicals from a common class or only on mixtures of chemicals that display identical mechanisms of action.

The assumption is that mixtures of chemicals that act via different mechanisms of action will not interact to produce adverse effects above those produced by each individual chemical — an assumption not supported by evidence. New laboratory results appear to indicate that the approach of assessing risk from mixtures of chemicals needs to be broadened beyond chemicals from a single class or chemicals that act by the same mechanism of action.

So far the impact of the research on mixture effects has had limited implications for risk assessment and regulatory action. In the EU, regulation concerning maximum residue limits of pesticides from 2005 states that it is important to make a further effort to develop a methodology, taking into account the cumulative and synergistic effects. The European Commission plans to publish a Communication on combination effects of chemicals in 2012 to address the issue of regulation of chemical mixtures in the EU.

In the USA, the EPA is working on several cumulative risk assessments on single classes of pesticides that act via a common mechanism of action. A toxic equivalency factor (TEF) approach (the outcome is similar to dose addition modelling) has been applied, similar to that used for dioxins and related compounds with relative potencies derived from *in vitro* and *in vivo* short-term assays. At this point, discussions have begun among laboratory scientists and risk assessors on how to deal more broadly with chemicals that act via different mechanisms of action, but no regulatory action has yet been taken on the question. It is expected that this will become a very active research and risk assessment area in the future.

Overall, rather predictable dose-additive relationships between the endocrine-disrupting potency of individual, similarly acting, chemicals and the ways in which they act together have been demonstrated, and if future research confirms these findings, powerful tools for prospective risk assessment will become available. These tools could show the way forward to make productive use of existing single chemical databases for the prediction of mixture effects.

3.3.4 The nervous system: Behavioural studies, neurodevelopment and thyroid effects

In addition to the effects of hormones on growth, puberty, reproductive function and development, the function of the nervous system is affected by hormones during development and adult life. Although vertebrate species differ greatly in how the nervous system is organised and activated by hormones, it seems clear that normal development and function are regulated to varying degrees by androgens, oestrogens and thyroid hormones. Since these hormones can play a critical role in the normal development of the nervous system, disruption of the hormone signals can affect nervous system function and related behaviours profoundly and permanently. The interactions between brain development and thyroid hormone have been reviewed (Ahmed et al., 2008), as have the foetal mechanisms involved in neurodevelopmental disorders (Connors et al., 2008). Neuroendocrine disruption was recently reviewed by Gore (2010); the neuroendocrine system is vulnerable to disruption because it is positioned at the juncture between the nervous system and the endocrine system. While there are numerous studies of the effects of EDCs on the nervous system and behaviour, most of the focus has been on the effects of hypothyroidism on nervous system function in humans. In contrast, animal studies on oestrogenic and androgenic effects on behaviour and nervous system function have received much less attention to date. There are several reasons for the lack of attention in this area.

1. The complexity of nervous system development and function.

2. Lack of understanding of how to extrapolate effects from laboratory species to humans, due to large species differences in sexual dimorphisms.

3. Many laboratory studies are conducted using irrelevant routes or exposures or only very high dose levels.

4. Lack of standardisation of behavioural methods and methods for nervous system evaluation.

In spite of the current shortcomings, one should be aware that hormones are critical in the development of nervous system function in all species including humans, and the effects of early exposure could be latent for decades. The need for test development in this area is apparent (Weiss et al., 2008), given that even those chemicals identified as neurotoxic in adults have mostly not been tested for developmental toxicity *in utero*.

3.3.5 Low-dose effects of EDCs

Both public and environmental health standards have historically relied on a central dogma: toxicant effects that do not occur at high levels of exposure to a chemical cannot be induced at much lower levels of exposure. Curves plotting response against dose are therefore monotonic and never reverse from positive to negative or vice versa. Studies with endocrine-active chemicals contradict this expectation; there is a growing body of evidence indicating that while a chemical's effect over a certain dose range may decrease as the dose is reduced, at very low doses the effect may actually become greater for a variety of sound biological reasons (e.g. see Gualteri et al., 2010 and Bouskine et al., 2009) resulting in non-monotonic dose–response curves. It is this definition of a 'low-dose' effect (encompassing the concept of the non-monotonic dose–response curve) that has caused much controversy over the last decade. Other definitions of 'low-dose effects' including those encompassing adverse effects occurring at 'low' environmentally relevant concentrations of an extremely potent chemical or below those doses typically used in standard toxicity testing are also relevant. This applies particularly to pharmaceuticals where effects have been shown to occur at part per trillion concentrations in wild fish; however, this subject will not be addressed at this point.

Instead, we will discuss what is probably the most controversial health concern that has been related to EDCs over the last decade: whether low-dose exposures to the environmental oestrogen BPA can promote human reproductive diseases and hormone-dependent cancers.

Controversy surrounding this question arose in the late 1990s, when a number of studies reported — for the first time — that BPA exposure at doses below the presumed NOAEL resulted in effects on the male reproductive endocrine systems of exposed rodents (Colerangle and Roy, 1997; Nagel et al., 1997; Steinmetz et al., 1997; Vom Saal et al., 1998). Two studies designed to repeat the vom Saal studies failed to find effects (Cagen et al., 1999; Ashby et al., 1999); since industry had funded the research, it was claimed that the results were biased. In response to this, the National Toxicology Program (NTP) in the USA conducted an open peer review of the literature in order to evaluate the scientific evidence on reported low-dose effects

of BPA (NTP, 2001). The review concluded that low-dose effects could be observed in some studies and not in others, but that pharmacokinetic data on BPA (such as bioavailability and half-life) were too scarce to strengthen or dismiss the plausibility of its low-dose effects. It also concluded that additional research was necessary to resolve the low-dose question.

In the last 10 years, much of this research has been conducted, but new questions have also arisen, reinforcing the same overall conclusion: that the 14 study outcomes funded by chemical industries contradict the 202 out of 217 government-funded studies reporting that low doses of BPA cause harm.

The most contentious issue is that the current NOAEL for BPA used for regulatory purposes in Europe and the USA is 5 mg/kg/day, based on the results of two studies in rodents (Tyl et al., 2002 and 2008). In many studies, effects of BPA such as changes in organ weight or tissue architecture, altered receptor expression, altered hormone concentrations, altered puberty and behavioural effects have been found in a range of two to three orders of magnitude below this. As this is within the range of human exposure levels (Richter et al., 2007), many scientists believe there is a risk to humans of adverse health effects.

The questions posed are related to the biological significance of the reported effects, the robustness of the studies, and whether these effects can be interpreted as adverse, thereby leading to a revision of the current NOAEL of 5 mg/kg and a more conservative risk assessment of BPA (Beronius et al., 2010). Vastly differing views on the significance of the toxicity of low doses of BPA have been held.

The data gaps mentioned in the 2001 NTP report have remained largely unaddressed, although new data have emerged on pharmacokinetics of BPA in mice, monkeys and women (Taylor et al., 2010), together with data suggesting that BPA can be absorbed through the skin. This would explain why levels found in the general population appear to be higher than theoretical doses received through food and drink (Zalko et al., 2011). Moreover, new studies reporting that BPA may affect neurodevelopment (Leranth et al., 2008; MacLusky et al., 2005) and increase prostate (Prins et al., 2010) and breast (Durando et al., 2007) cancer risks have also been published), and formed the basis of the updated NTP report expressing some concern because of these findings (NTP 2008); these endpoints were not assessed in the studies of Tyl et al. (2002 and 2008).

Taken together, the many studies on BPA serve to illustrate that extrapolating from high-dose animal data to low-dose human exposures is perhaps not an appropriate approach for EDCs. In many cases, the presence or absence of a low-dose effect is compromised by the statistical power of the experimental design, the lack of a full dose-response curve and the spread or absence of data points in the low-dose range.

3.3.6 Screening and testing programmes for EDCs

When concerns arose in the late 1990s over EDCs in the environment, several national and regulatory entities initiated efforts to determine how to deal with the potential problem. In general, screening assays include both *in vitro* and short-term *in vivo* assays designed to detect endocrine activities, but not necessarily while using the relevant routes of exposure or establishing dose responses or ascertaining if the effects are adverse.

Positive screening results, determined by a weight-of-evidence evaluation, would then be tested in multigenerational tests that would establish dose responses and the adverse nature of the effects, use relevant routes of exposure and include the most sensitive life stages. It also has been proposed that equivocal positive screening assay results are re-evaluated more thoroughly before extensive resource (animal, time etc.) testing is initiated (Gray et al., 2001).

Current screening efforts have focused on development and validation of assays to detect oestrogens, androgens and thyroid active substances. The screening battery also is intended to detect inhibition of steroid hormone synthesis and — in the USA —alterations of hypothalamic-pituitary function that result in altered hormone levels.

In vitro assays under consideration or adopted include ER and AR binding assays, functional assays with cell lines to distinguish agonists from antagonists and cell lines to assess steroidogenesis. Since *in vitro* assays can produce false positives and false negative results, as they do not account for absorption, distribution, metabolism and excretion, *in vivo* assays that eliminate these problems are necessary.

Short-term *in vivo* assays include detection of oestrogenic activity in the uterotrophic assay and androgenic and anti-androgenic activity in the Hershberger assay. The US EPA is also using

alterations of pubertal development in weanling male and female rats, since these models should be sensitive to all the above endocrine modes of action.

Short-term screening and longer term assays for EDC effects in lower classes of vertebrates (fish, frog, etc.) are also being developed. Proposed screening assays are now being validated in interlaboratory studies to determine the sensitivity, specificity and reproducibility of the assays using standardised protocols. The following discussion will first highlight the activities in Europe and then those in the USA.

The OECD undertook to revise existing guidelines and develop new guidelines for the screening and testing of potential EDCs in 1997 (OECD, 1998). This activity is managed by a Validation Management Group (VMG) reporting to the Endocrine Disruptors Testing and Assessment (EDTA) Task Force as part of the OECD Test Guidelines Programme.

One portion of the activity involves validating the rodent uterotrophic and Hershberger bioassays, as *in vivo* screens intended to identify compounds that are suspected agonists or antagonists, and to assist the prioritisation of positive compounds for further evaluation. In the multiphase validation programmes, standardised protocols were developed and successfully tested against the high-potency reference substances, as were less potent man-made chemicals. Both protocols were robust, reproducible, and transferable across laboratories using these reference compounds. The OECD Test Guidelines have been written for these two; the other *in vivo* screening assays are progressing, but the validation process has not yet been completed.

The US EPA was given a mandate in 1996 under the Food Quality Protection Act and Safe Drinking Water Act to develop a screening and testing programme for endocrine effects. Some of the impetus for these actions arose in 1991 from a work session entitled 'Chemically induced alterations in sexual development: the wildlife/human connection' (Colborn et al., 1992), which stated that many compounds introduced into the environment by human activity are capable of disrupting the endocrine system of animals, including fish, wildlife, and humans.

In response to the 1996 legislative mandate for an endocrine screening and testing programme, the US EPA formed the EDSTAC, which proposed a tiered screening and testing strategy for EDCs in its final report (1998). The EDSTAC proposal includes the following:

(i) a process to prioritise chemicals for evaluation;

(ii) tiered screening (Tier 1); and

(iii) testing (Tier 2) batteries.

The chemical universe to be considered includes over 80 000 chemicals, of which only a subset of high-priority chemicals would initially enter the screening programme. Prioritisation would include an estimation of the chemical's ability to interact with steroid hormone receptors, using computational approaches such as quantitative structure–activity relationships (QSARs) (e.g. Liu et al., 2006) for chemicals that bind steroid receptors or HTPS using hormone-dependent gene expression assays, among several other factors. An important ongoing task is to develop validated QSAR models or HTPS sufficiently rigorous to meet the needs of the regulatory agencies for an initial rapid screening for endocrine-disrupting effects.

Currently the US EPA is responsible for developing and implementing the EDC screening and testing battery in the USA. Although implementation of the battery was initially planned for 2004, EPA announced the initial list of chemicals to be screened and the availability of the Tier 1 screening battery of 11 validated assays and related test guidelines in 2009. Many activities exceed their target completion dates by about 4.5 to 6 years. Consequently, 14 years after passage of the Food Quality Protection Act (FQPA) and Safe Drinking Water Act amendments, EPA's Endocrine Disruptor Screening Program (EDSP) has not determined whether any chemical is a potential endocrine disrupter.

Many of the efforts of the US EPA and OECD have been coordinated between the two agencies in order to conserve resources and facilitate the development of the assays. Considerable research effort is going into validating these assays and to developing new *in vitro* assays. Several issues need to be addressed, including detecting additional endocrine mechanisms of action. Apart from the ERs, ARs and thyroid hormone receptors (TRs), there are no *in vivo* tests adopted for other receptors and a significant amount of work on assay development and standardisation will need to be completed before adoption of these tests is envisaged.

There is also a need to determine if using multiple assays for the same activity might improve the sensitivity of screening batteries and if *in vitro* assays from non-mammalian vertebrates are required to detect species-specific EDCs. In addition, EPA's HTPS effort failed when it was first

proposed 10 years ago, because there were few, if any, hormone-dependent gene expression assays available. Since this is no longer the case, and ER, AR and TR reporter gene assays are available, HTPS systems are now being implemented for prioritisation and screening efforts.

Research is also needed in the testing arena. The current multigenerational assays need to be enhanced to include all the endpoints sensitive to oestrogens, androgens and thyroid hormones, and we now have enough data to improve multigenerational experimental designs to obtain greater statistical power from fewer animals. The use of enhanced multigenerational test protocols also would greatly reduce the likelihood that a multigenerational test would need to be repeated because it failed to include the right endpoints or detect a NOAEL.

3.3.7 References

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3.4 Exposure, risk and policy

3.4.1 Introduction

Over the last 10 years, endocrine disrupter research has uncovered a host of issues that challenge some of the premises of traditional risk assessment procedures for chemicals. These issues include the realisation that simultaneous exposure to multiple EDCs may produce combination effects below effect thresholds, as well as the growing evidence for non-monotonic dose–response curves, often at low doses. Of considerable concern is that established risk assessment methods were incapable of anticipating the impact of EDCs. As a result, the effects of many EDCs on wildlife only became apparent after significant responses had already occurred. There are similar problems with conventional toxicity testing procedures used for evaluations of human health risks, and these procedures are now considered to be inadequate to detect EDCs.

Some characteristics typical of EDCs make the application of traditional risk assessment processes difficult. It is well recognised that EDCs may exert their effects during specific life stages (for example *in utero* or during puberty), but the health consequences of these interactions may become apparent only later in life. This delay in the manifestation of effects can span several decades, thus considerably complicating any attempts to establish causal relationships between exposure and effect.

Another issue is the use and interpretation of methods for the identification of EDCs. These rely mostly on 'mode of action' screens (*in vitro* and *in vivo*) that are usually based on detecting interactions of EDCs with steroid receptors, commonly the oestrogen, androgen and thyroid receptors, or with hormone metabolising enzymes. However, the relevance of these screening endpoints to endpoints of toxicological importance is restricted

in many cases, and for this reason, the screening test endpoints themselves are often of limited value for risk assessment purposes. Assays that capture features of conditions relevant in humans are either not available or too costly to run for screening purposes.

The traditional response to this problem has been to state that endocrine activity should not be considered as an endpoint in its own right, but as a mechanism of action that could potentially lead to adverse outcomes including carcinogenicity, developmental and reproductive toxicity, teratogenicity, immunotoxicity and neurotoxicity. The hope is that such effects can be captured by toxicity testing, but recently, limitations have become apparent. Until evidence from more refined bioassays is forthcoming, something not foreseeable in the immediate future, ways have to be found to productively utilise information from screening endpoints for risk assessment purposes, despite their shortcomings.

This document is intended to summarise some of the issues that challenge traditional risk assessment methods, and to review those that present complications. It is not meant to be comprehensive. Rather, the aim is to highlight key developments and to reflect on how these might be utilised to improve the ways in which EDCs are dealt with in the EU and other parts of the world.

3.4.2 Issues for exposure assessment, hazard characterisation and risk evaluation

Before considering EDC-specific issues that impact on risk assessment, it may be helpful to briefly outline the elements of chemicals risk evaluation processes as practiced in the EU.

(1) Exposure assessment aims to estimate or measure the amount of a chemical that can be taken in by humans or wildlife. It also involves considering sources of exposure, and routes and pathways of distribution, as well as evaluations of bioavailability. This information is essential for the development of effective risk management strategies aimed at reducing exposures. In environmental exposure assessment, information about pathways of dispersion is used to estimate predicted effect concentrations (PECs) in water, soil and other media.

(2) Hazard characterisation is ultimately intended to derive a measure of a chemicals dose that can be tolerated without health risks. In human hazard assessment, such doses are variously referred to as acceptable, TDI, acceptable daily intake (ADI) or HLVs. In environmental risk assessment, the equivalent dose measure is the predicted no-effect level (PNEC).

    HLVs are usually arrived at on the basis of data from animal experiments where doses without noticeable effect, NOAELs, are estimated. To convert these estimates into doses deemed to be safe for humans, so-called safety or assessment factors are applied. These factors are intended to take account of species–species extrapolations and other sources of uncertainty.

(3) Finally, an attempt is made to assess risks to humans or wildlife by comparing exposure levels with measures of doses deemed to be safe: in human risk assessment, the HLV; in environmental risk assessment the PNEC. The ratio between exposure and HLVs, and in the environmental arena, of PECs and PNECs, is then used as the trigger value for deciding whether regulatory action should be taken. Ratios smaller than one are deemed to signal absence of risk.

a) Combination effects

The risk assessment process outlined above is usually carried out with single chemicals and does not take account of the possibility of combination effects which might occur when humans or wildlife come into contact with several agents simultaneously. It is often claimed that assessment factors cover such mixture effects, but there is still relatively limited information about the ways in which chemicals may act together.

Considering that there are between 30 000 and 50 000 chemicals marketed in the EU, and an estimated 50 000 chemicals present in surface waters (Matthiessen and Johnson, 2006), the general potential for mixture effects is considerable.

Recent research with EDCs has shown that certain classes of EDCs can act together (for a review, see Kortenkamp, 2007). There are several noteworthy demonstrations of combination effects: of multiple oestrogenic chemicals in the rat (Tinwell and Ashby, 2004), and in fish (Brian et al., 2005); as well as of anti-androgens (Gray et al., 2006; Hass et al., 2007; Howdeshell et al., 2008; Rider et al., 2009; Christiansen et al., 2009) and TDCs (Crofton et al., 2005) in rodents. Contradicting earlier concerns about

synergisms with mixtures of EDC, the evidence available to date shows that classes of similarly acting EDCs (e.g. oestrogenic, anti-androgenic agents) produce additive mixture effects. However, it remains to be seen whether this also applies to combinations of EDCs with diverse modes of action.

Considering that exposure to EDCs usually involves multiple chemicals, additivity is a matter of concern. This is highlighted by demonstrations that EDCs, even when present at low, apparently ineffective levels, are able to produce significant (additive) effects when applied as a mixture (Silva et al., 2002; Rajapakse et al., 2002; Tinwell and Ashby, 2004, Brian et al., 2005; Crofton et al., 2005). Thus, PNECs or NOAELs derived for single chemicals may underestimate hazards when exposure relates to a large number of similarly acting chemicals simultaneously. Whether or not combination effects will occur in such situations depends on the number of chemicals that also produce the effect of interest, as well as their levels.

What undermines the traditional risk assessment paradigm of a threshold dose below which a chemical fails to produce effects is the realisation that every similarly acting chemical in a combination contributes to the overall mixture effect, in proportion to its potency and dose. Whether the individual doses are also effective on their own is immaterial in such situations, and consequently even doses below thresholds are of relevance (Kortenkamp et al., 2007; Kortenkamp, 2008). This is of importance in situations where EDCs act together with endogenous hormones. Since there is already endogenous biological activity, the effect of even very small exposures is not zero. Whether the resulting additional effects are significant depends on the potency, level and number of EDCs. This also means that the effects of EDCs cannot always be dismissed with the argument that their potency in relation to endogenous hormones is low. Thus, the idea of threshold doses begins to lose its relevance when dealing with EDC mixtures.

b) 'Known unknowns' — the need to search for EDCs in exposure assessment

The above mixture effect issues have important implications for EDC exposure assessment. If every similarly acting EDC can contribute to combination effects even at doses below effect thresholds, then it is essential to have comprehensive information about the array of EDCs that contribute to human and wildlife exposure.

Although considerable progress has been made in identifying EDCs, our knowledge about the nature of EDCs relevant for human and wildlife exposure scenarios is still incomplete. EDCs need to be identified in terms of their biological effects, before chemical analytical methods can be brought to bear. Since analytical methods are only useful when it is clear what should be analysed, there is much potential for missing chemicals whose endocrine activity has not yet been defined.

One way of overcoming these problems has been to use TIE schemes that combine chemical extraction and fractionation with *in vitro* bioassays for the identification of bioactive fractions, with the ultimate aim of pinpointing relevant EDCs. This approach has worked well with the yeast oestrogen screen and oestrogenic chemicals in surface waters (Desbrow et al., 1998) and riverine sediments (Peck et al., 2004). It has led to the identification of equine oestrogens from pharmaceutical products used in hormone replacement therapy for the alleviation of menopausal symptoms (Gibson et al., 2005).

There is little experience with other *in vitro* assays representative of hormone synthesis, transport and degradation. No work has been carried out with extracts from human tissues or body fluids. TIE schemes can be very powerful in highlighting the presence of bioactive, but as yet unidentified, EDCs.

Furthermore, there is an abundance of data relating to the levels of specific EDCs in human tissues and environmental media, but these data were gathered in analytical programmes that focused on one, or on a limited number, of chemicals (see the compilation of data in WHO, 2002).

Exposure assessment schemes that directly address the mixtures issue by analysing a range of chemicals in one and the same sample are relatively few and far between (an exception are the National Nutrition and Health Survey studies conducted by the US Center for Disease Control (CDC) in 2001, 2003 and 2005, and the WWF programme where several prominent politicians and EU officials gave blood for the analysis of a range of environmental chemicals (WWF, 2004); another example is Wolff et al.'s work (2006)). Very recently, a comprehensive analysis of hormonally active pollutants that occur together in mothers' milk has been conducted (Schlumpf et al., 2010).

Due to the wide-ranging and diverse uses of EDCs, exposure scenarios are quite complex. The challenge of obtaining a comprehensive picture of the exposures can only be met by adopting integrated

approaches that take account of cumulative exposures through multiple routes, including food, inhalation and dermal uptake. Traditional exposure assessment schemes, with their focus on individual chemicals, are likely to fail in this respect.

Further attempts should be made to adopt biomonitoring approaches for exposure and risk assessment (see, for example, Albertini et al., 2006). The EU has funded research to harmonise human biomonitoring in Europe through the ongoing large-scale COPHES and DEMO-COPHES projects. Material from environmental and human biobanks will become invaluable to fill the data gaps that hamper better EDC exposure assessment. There is a growing realisation that polar contaminants that are subject to hydrolysis can degrade rapidly in blood samples; serum samples or urine is required to avoid underestimation of exposure to such chemicals. This may limit the usefulness of human tissue samples collected in some epidemiological studies.

c) Dose-time relationships, non-monotonic dose–response curves and the low-dose issue

Another basic premise of traditional risk assessment procedures is the idea that long-term effects at low levels of exposure can be anticipated by extrapolation from tests conducted at high doses with comparatively short exposure durations. Matthiessen and Johnson (2006) have pointed out that acute-to-chronic ratios for many non-EDCs and non-carcinogens rarely exceed 100-1 000. In other words, long-term effects normally fail to materialise at doses (or concentrations) below 0.1 % to 1 % of those obtained in short-term tests.

However, this is not fulfilled with some EDCs, the synthetic oestrogen EE2 with an acute-to-chronic ratio of more than 1 million in fish serving as a striking example. There is little information about the relation between dose and exposure duration, and about whether loss of effects due to lowering of the dose can be compensated by extending exposure duration.

Observations of non-monotonic dose–response relationships for some EDCs and specific endocrine endpoints present another challenge. Concerns have been voiced that conventional testing regimes may overlook effects that are present at low doses, but vanish as doses increase (vom Saal and Hughes, 2005). However, because of purported difficulties with reproducing some of these findings, sometimes even within the same laboratory, this topic has triggered an unusually heated debate, with claims of bias attributed to sources of research funding (vom Saal and Hughes, 2005).

Ashby et al. (2004) have suggested that variations in control responses might be at the root of these reproducibility problems, but this was dismissed by vom Saal and Hughes (2005) with the argument that background variability in control responses does not in itself invalidate experimental observations, as long as the experimental system is shown to remain sensitive to positive control agents utilised for the effect in question.

To complicate matters further, there are many instances where researchers attempted to replicate findings using different animal strains, feeds, etc., thereby introducing the potential for systematic errors (for some recent examples, see Ohsako and Tohyama, 2005; vom Saal and Hughes, 2005; and Oehlmann et al., 2006). Another issue is that many 'low-dose' EDC studies have employed too few dose levels, falling short of quality criteria laid down by bodies such as the OECD.

Despite these disputes, observations of low-dose effects and non-monotonic dose–response curves continue to appear. An example is the oestrogenic UV filter substance 3-benzylidene camphor, which induces increased embryo production in aquatic snails at low, but not at high, doses (Schlumpf et al., 2004). An explanation for this effect may lie in dose-dependent changes in the mode of action of the chemical, such that oestrogenic effects are masked by toxicity at higher doses.

Similar conclusions have been drawn from experiments with phytoestrogens (Almstrup et al., 2002), and multiple mechanisms are likely to be involved with EDCs. This would suggest that extrapolations from short-term experiments at high doses to lower doses are fatally flawed.

Attention has been drawn to another, previously neglected phenomenon. By exploiting the statistical power afforded by a high-throughput *in vitro* assay, the E-Screen, Silva et al. (2007) were able to demonstrate that many oestrogenic agents exhibit very shallow dose–response curves in the low effect range.

Due to biological variation and limited statistical power, very small effects are not normally measurable in *in vivo* assays, where responses as high as 10 % to 20 % often cannot be distinguished from untreated controls. However, if such shallow dose-response curves also exist with *in vivo* ED

endpoints, the limited resolving power of such assays may lead to serious underestimations of effects during the hazard identification step of risk assessment.

d) Delayed action and specific life stages of increased vulnerability

As Sharpe and Irvine (2004) have emphasised, exposure *in utero* probably poses the greatest risks for adverse reproductive health effects from EDCs, in males. Such risks may only manifest many years later in adult life, long after the putative causative agents have disappeared. Similar delays between exposure and disease manifestation also exist for conditions affecting women, as illustrated by the recent demonstration of increased breast cancer risks among women exposed to DES during their foetal life (Palmer et al., 2006).

The availability of serum samples collected during young women's puberty has made it possible to demonstrate associations between DDT metabolites and breast cancer later in their lives (Cohn et al., 2007). Another example of delayed action by hormonally active chemicals can be found in a common fish species, the roach (*R. rutilus*). As a result of exposure to oestrogens from STWs, many roach populations experience endocrine-disrupting effects, including the appearance of eggs in testes of male fish. Since these ovotestes are not present in newly mature fish, it is thought that the phenomenon is caused during larval development, only to become more intense as fish age (Jobling et al., 2005).

Considering that hormones not only act as messenger substance for the maintenance of key life functions, but are also important triggers during development and sexual differentiation, such phenomena are not surprising. However, these examples highlight how important it is to take account of vulnerable life stages during the identification of risks potentially associated with EDCs (Birnbaum and Fenton, 2003).

These features of hormonally active chemicals pose perhaps the most serious challenges in terms of exposure and risk assessment. If exposure assessment strategies are to capture potentially causative agents, monitoring has to take place during these windows of vulnerability, and not when diseases have manifested. Monitoring during later life stages is likely to produce a warped picture in terms of risk assessment, with risks likely to be overlooked.

All these factors enormously complicate any attempts to establish a causal relation between exposure and effect, and there are serious doubts as to whether causality can ever be established for human populations under these circumstances. For these reasons, it is crucial to make use of weight-of-evidence approaches in order to gain rational criteria for the level of proof that is deemed necessary to trigger regulatory action.

e) Species differences

An issue particularly relevant to ecological and environmental risk assessment is the need to extrapolate from test results obtained with one species, to entire ecosystems. Given that many EDCs interact with hormone receptors that are highly conserved through evolution, it may seem that this task is extremely promising. Indeed, there are similarities, for example, in the feminising effect of oestradiol on almost all vertebrate species, but these are qualitative in nature.

Perhaps due to interference with other metabolic processes, the doses and concentrations at which effects occur in vertebrate species vary widely, and extrapolations from species to species, or even from strain to strain, let alone to humans, must be made very cautiously. In invertebrates, the assessment of endocrine effects is currently hampered by knowledge gaps about their endocrinology (see Section 3.2).

3.4.3 Testing, screening and implications for risk assessment and policy

It is widely recognised that current international testing schemes were not designed for EDCs and are deficient in estimating EDCs' likely impact on human health and wildlife. In response to these problems, considerable efforts have been made by the OECD and other organisations (FRAME, ECVAM) to expand test development to include procedures responsive to EDCs. This section briefly summarises issues and challenges for EDC test development, and explores options for dealing with knowledge gaps and uncertainties in risk assessment and regulation.

Testing and screening

A wide variety of assays are available for the identification and hazard characterisation of EDCs, ranging from *in vitro* assays to multigenerational and full life-cycle tests (see, for example, OECD,

2002). Nevertheless, EDC testing and screening faces a dilemma over hazard characterisation: given that many EDCs act during specific developmental periods, an identification of the entire spectrum of EDC effects would require multigenerational studies and full life-cycle testing. However, to subject every suspect chemical to such exhaustive testing is not sustainable due to time and cost constraints. It is also untenable from an animal welfare point of view.

In contrast, most of the available *in vitro* and *in vivo* screening assays can be conducted rapidly, but they only capture interactions with the oestrogen, androgen and thyroid receptors and consequent downstream events, as well as interference with enzymes important for hormone synthesis and degradation. Although positive test outcomes indicate the potential for ED, the relevance of screening endpoints for risk assessment is often limited.

For example, key processes of male sexual differentiation are regulated by the AR, and after exposure *in utero*, AR antagonists may lead to feminising effects among male offspring. However, not all AR antagonists identified in *in vitro* screens are capable of inducing such effects *in vivo*, possibly because the tissue concentrations attainable *in vivo* are too low to trigger effects, due to metabolic processes that cannot be captured in *in vitro* assays.

Similar problems exist with the interpretation of positive results from oestrogenicity screening, both *in vitro* and *in vivo*. The concern is that oestrogens may contribute to breast cancer risks, but they also play a role in normal breast development. Although perhaps indicative of a potential for endocrine effects, it is difficult to equate oestrogen receptor activation as such with adversity, because dose levels, biological context and timing are key issues.

The appearance of ovotestes in fish is a phenomenon mediated by oestrogen receptor activation, but other processes such as antiandrogenicity also play a role (Jobling et al., 2009), thus complicating the interpretation of positive results from oestrogen screening assays.

To complicate matters further, there are currently no validated test systems for many human conditions of concern. Although some features of the TDS have been described in an experimental model in the rat (Fisher et al., 2003), it is not possible to recapitulate testis cancer in rodents.

Similarly, certain oestrogenic chemicals can advance duct development in the mammary gland, with possible implications for breast cancer risks (Durando et al., 2006), but these effects are not normally captured in carcinogenesis bioassays. Certain chemicals are capable of inducing mammary carcinoma in the rodent strains used in the US NTP for carcinogenesis testing, but these neoplastic events bear little relation to the processes that determine breast cancer in women. This complicates the interpretation of results from these rodent models. Consequently, rodent mammary carcinogens are not predictive of chemicals likely to contribute to breast cancer in women (Rudel et al., 2007).

a) Implications for regulation and policy

The strategy followed to deal with these difficulties, by the OECD for example, is to promote the development of short-term assays that might act as triggers for more comprehensive testing (for environmental testing, see Hutchinson et al., 2003). However, test guideline development and validation is a time-consuming process, and an adequate set of tests will not be available for many years. Meanwhile, concerns about EDCs are such that it would be unacceptable if the long time-frame for assay development seriously delays or even blocks regulatory action. It is therefore necessary to develop a risk assessment and regulatory framework for dealing pragmatically with incomplete knowledge about EDCs. Several interesting proposals for such frameworks have been made (WWF, 2002; Hass et al., 2004; Matthiessen and Johnson, 2006). Although these differ in certain details, there is agreement on several important principles.

1. Putative EDCs need to be dealt with on a case-by-case basis, which requires expert judgement.

2. Positive outcomes from *in vitro* and *in vivo* screening assays should generally trigger further testing in long-term assays, and *in vivo* testing is needed for more definite identifications of EDCs. However, this leaves the issue of false negative outcomes unresolved, which leads to major problems in terms of overlooking EDCs.

3. Because screening assays only capture certain aspects of endocrine action, negative outcomes cannot be taken as evidence for absence of endocrine activity by other mechanisms.

4. Data from *in vivo* screening assays (e.g. uterotrophic assay, Hershberger assay) should be used for preliminary risk assessment purposes. This is justified because these assays indicate endocrine activity with relevance to

humans and wildlife, and because the potency of EDCs in screening assays is often comparable with that in long-term studies (Hass et al., 2004). However, in view of their short-term nature, estimates from screening assays should be combined with larger-than-usual assessment factors.

b) Hazard classifications

Hazard classifications are a key element of the new EU REACH chemicals legislation. For chemicals of high concern, such as chemicals carcinogenic, mutagenic or toxic to reproduction (CMR), and persistent, bioaccumulative and toxic (PBT), or very persistent and very bioaccumulative (vPvB) substances, authorisation will be required before they can be marketed.

The European Commission's White Paper *Strategy for a future Chemicals Policy* states that the majority of EDCs would have to undergo authorisation under REACH, and consequently, EDCs are currently classed as substances giving rise to concerns similar to those classed as CMR. However, difficulties have arisen in defining which EDCs should be brought into authorisation and which criteria should be used to make such decisions. While the health effects associated with some EDCs may fulfil the criteria laid down for carcinogens and reproductive toxins, only a small fraction of the chemicals listed by the European Commission as endocrine disrupters ('BKHlist') are classed as carcinogens or reproductive toxicants. For example, BPA and nonylphenol would not automatically qualify for authorisation, because they are currently classified as low-category reproductive toxicants.

The situation could be improved by introducing a new chemicals grouping for endocrine disrupters, as suggested by the WWF (2002). However, this may lead to complications, because 'endocrine disruption' is not an effect, but a mode of action. Alternatively, attempts could be made to subsume EDCs into the group of reproductive toxicants by making extended use of data from endocrine disrupter screening and testing. However, it is acknowledged that this categorisation might be too limiting, as our knowledge about ED expands to include non-reproductive effects and non-reproductive hormones. With great caution, and on a case-by-case basis, decisions about placement into categories could be handled as follows (Hass et al., 2002).

1. In cases where it is debated whether reproductive and developmental toxicity should be considered adverse, evidence from *in vitro* and *in vivo* screening can be used to demonstrate that chemicals work by a mechanism relevant to humans. This information can support upgrading to categories that trigger authorisation.

2. Positive results from *in vivo* screening assays such as the uterotrophic assay or the Hershberger assay may be used directly for classification, and in specific cases it may be warranted to require authorisation for such chemicals until further test data become available to dispel concerns.

3. Chemicals where only *in vitro* data are available are not currently classified. But positive *in vitro* test outcomes should trigger entry into the EU list of potential EDCs. These substances should be prioritised for further testing.

It should be noted, however, that highly bioactive chemicals will not only show reproductive and developmental toxicity, but are likely to be immunotoxic, developmental neurotoxicants and perhaps even carcinogens.

c) Mixture effects — implications for risk assessment and regulation

It is evident that the traditional chemical-by-chemical approach to risk assessment is inadequate when dealing with EDCs (and chemicals with other toxic profiles). The biological reality of combination effects from exposure to multiple agents at low doses highlights the potential for underestimating risks when mixture effects are not taken into account.

This underlines the need to modify current risk assessment practice, if humans and the environment are to be protected adequately from multiple exposures to endocrine disrupters. As a first step in the direction of implementing better risk assessment, the idea of grouping endocrine disrupters according to suitable similarity criteria comes to mind, as is already common practice with the group-wise assessment of aryl hydrocarbon receptor (AhR) agonists such as PCDDs, PCDFs and PCBs in the TEF/TEQ approach. For example, in an opinion paper, the European Commission's Scientific Committee on Toxicology, Ecotoxicology and the Environment (SCTEE, 2004) pointed out that 'for compounds with identical mode of action, such as oestrogenic hormones and xenoestrogens … the performance of individual risk assessments is problematic […] The effects may be additive,

especially since these chemicals co-occur in the aquatic environment'.

However, the challenge lies in defining what 'identical modes of action' could mean for EDCs, and how this could be translated into workable criteria for grouping endocrine disrupters according to 'similar modes of action'. One suggestion would be to group endocrine disrupters according to the steroid receptors with which they interact. Thus, all oestrogens, androgens, anti-androgens, etc. could be regulated together.

However, in taking this approach, the criteria chosen for grouping should be considered carefully. Too narrow a focus on molecular mechanisms might lead to problems and prove unworkable. The issue can be illustrated by taking anti-androgens as an example. With a narrow focus on 'identical modes of action', all AR antagonists could be considered, but this would leave out agents that are able to disrupt male sexual development by interfering with foetal steroid synthesis, such as certain phthalates. Thus, application of a phenomenological similarity criterion ('all agents that disrupt male sexual development by disrupting androgen action in foetal life through a variety of mechanisms') would serve the group of anti-androgens better. The US National Research Council has advocated such an approach for the grouping and risk assessment of phthalates and other anti-androgenic chemicals (NRC, 2008). Kortenkamp and Faust (2010) have considered the data requirements for adopting more holistic grouping criteria for phthalates and anti-androgens, and have conducted a risk assessment.

The evidence that groups of oestrogenic, anti-androgenic and TDCs act together in an additive fashion cannot be ignored. One suggestion for a way forward is to temporarily group endocrine disrupters together accordingly, and to subject these groups to common hazard and risk assessment, until better criteria for grouping become available. Great care should be taken not to apply inappropriately restrictive criteria in carrying out these classifications. Endocrine disrupters should be arranged according to their ability to provoke similar effects, rather than according to similar mechanisms of action. Given that the expectation of parallel dose–response curves is unrealistic, use of the TEF concept is impractical. Instead, dose addition (see Section 6.3) should be used for calculating quantitative additivity expectations.

In regulation, special consideration should be given to EDCs that act in concert with endogenous sex hormones. As discussed above, the assumption of threshold-mediated action does not apply to such chemicals, because every little dose quantum adds to an already existing effect. In such cases, it should be considered first whether there are alternatives that can substitute such EDCs. If substitution for certain uses is possible, regulatory action should be taken without further risk assessment. Examples where this approach may be viable include oestrogenic agents such as UV filter substances, parabens, certain synthetic musks and meat hormones.

3.4.4 References

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4 EU-funded research projects of relevance to the report ([1])

2-FUN	Full-chain and uncertainty approaches for assessing health risks in future environmental scenarios
ACE	Analysing combination effects of mixtures of estrogenic chemicals in marine and freshwater organisms
ANEMONE	Assessment of neurobehavioural endpoints and markers of neurotoxicant exposures
ARCRISK	Arctic health risks: impacts on health in the arctic and Europe owing to climate-induced changes in contaminant cycling
ATHON	Assessing the toxicity and hazard of non-dioxin-like PCBs present in food
BEEP	Biological effects of environmental pollution in marine coastal ecosystems
BIOCOP	New technologies to screen multiple chemical contaminants in food
BONETOX	Bone development and homeostasis-critical targets in toxicology. Research to support test-method development and human health risk assessment for dioxins and other endocrine-disrupting compounds in the food chain
CADASTER	Case studies on the development and application of in-silico techniques for environmental hazard and risk assessment
CAESAR	Computer assisted evaluation of industrial chemical substances according to regulations
CASCADE	CASCADE 'Network of Excellence' (NoE) became CASCADE 'Association for Collaboration in Endocrine Research and Training' (ACERT) in 2010
CLEAR	Climate change, environmental contaminants and reproductive health
CONFFIDENCE	Contaminants in food and feed: inexpensive detection for control of exposure
COMPARE	Comparison of exposure-effect pathways to improve the assessment of human health risks of complex environmental mixtures of organohalogens
COMPRENDO	Comparative research on endocrine disrupters — phylogenetic approach and common principles focussing on androgenic/antiandrogenic compounds
CONTAMED	Contaminant mixtures and human reproductive health — novel strategies for health impact and risk assessment of endocrine disrupters
COPHES	Consortium to perform human biomonitoring on a European scale. European coordination action on human biomonitoring
DEER	Developmental effects of environment on reproductive health
DEVNERTOX	Toxic threats to the developing nervous system: *in vivo* and *in vitro* studies on the effects of mixture of neurotoxic substances potentially contaminated food
DIOXIN RISK ASSESSMENT	Comprehensive risk analysis of dioxins: development of methodology to assess genetic susceptibility to developmental disturbances and cancer
EASYRING	Environmental agents susceptibility assessment utilising existing and novel biomarkers as rapid noninvasive testing methods
EDEN	Exploring novel endpoints, exposure, low-dose- and mixture-effects in humans, aquatic wildlife and laboratory animals
EDERA	Development and implementation of new '*in vivo*' and '*in vitro*' systems for the characterisation of endocrine disruptors
ENDISRUPT	Identification of critical rat testicular genes altered after fetal androgenic disruption by flutamide: use of DNA microarray
ENDOMET	Dysregulation of endogenous steroid metabolism potentially alters neuronal and reproductive system development: effects of environmental plasticisers
ENFIRO	Life cycle assessment of environment-compatible flame retardants (prototypical case study)
ENRIECO	Environmental health risks in European birth cohorts

([1]) For updates, see http://ec.europa.eu/research/environment/index_en.cfm?pg=health and http://ec.europa.eu/research/endocrine/index_en.html.

ENVIRON.REPROD.HEALTH	Increasing incidence of human male reproductive health disorders in relation to environmental effects on growth- and sex steroidinduced alterations in programmed development
ENVIROGENOMARKERS	Genomics biomarkers of environmental health
ESCAPE	European study of cohorts for air pollution effects
ESTROGENS AND DISEASE	Consequences of pre-natal exposure to low dose estradiol in C57Bl/6J as a model for environmentally induced endocrine disruption; identification of potential diagnostic markers for endocrine disruption
EURISKED	Multi-organic risk assessment of selected endocrine disrupters
EXPORED	Exposure-outcome relationships in male urogenital malformations with special reference to endocrine disrupters
F&F	Pharmaceutical products in the environment: development and employment of novel methods for assessing their origin, fate and effects on human fecundity
FACET	Flavours, additives and food contact material exposure task
FAMIZ	Food web uptake of persistent organic pollutants in the Arctic marginal ice zone of Barents Sea
FIRE	Flame retardant integrated risk assessment for endocrine disruption: risk assessment of brominated flame retardants as suspected endocrine disrupters for human and wildlife health
GENDISRUPT	Genetic markers and susceptibility to the effects of endocrine disruptors during mammalian testis development
HIWATE	Health impacts of long-term exposure to disinfection by-products in drinking water
INUENDO	Biopersistent organochlorines in diet and human fertility: epidemiological studies of time to pregnancy and semen quality in Inuit and European populations
MENDOS	BioMimetic optical sensors for environmental endocrine disruptor screening
NEWGENERIS	Newborns and genotoxic exposure risks: development and application of biomarkers of dietary exposure to genotoxic and immunotoxic chemicals and of biomarkers of early effects, using mother-child birth cohorts and biobanks
NORMAN	Network of reference laboratories and related organisations for monitoring and bio-monitoring of emerging environmental pollutants
NOMIRACLE	Novel methods for integrated risk assessment of cumulative stressors in Europe
OBELIX	Obesogenic endocrine disrupting chemicals: linking prenatal exposure to the development of obesity later in life
OSIRIS	Optimised strategies for risk assessment of industrial chemicals through integration of non-test and test information
PBDE-NTOX	Study the effects of developmental exposure to PBDEs in terms of neurotoxicity, namely neurobehavioural development and clarification of possible mechanisms of action
PCBRISK	Evaluating human health risk from low-dose and long-term PCB exposure
PERFOOD	Perfluorinated organics in our diet
PHIME	Public health impact of long-term, low-level mixed element exposure in susceptible population strata
PHYTOS	The prevention of osteoporosis by nutritional phyto-oestrogens
PHYTOPREVENT	The role of dietary phytoestrogens in the prevention of breast and prostate cancer
PIONEER	Puberty onset – influence of nutritional, environmental and endogenous regulators
REEF	Reproductive effects of environmental chemicals in females
REPROTECT	Development of a novel approach in hazard and risk assessment or reproductive toxicity by a combination and application of *in vitro*, tissue and sensor technologies
RISKCYCLE	Risk-based management of chemicals and products in a circular economy at a global scale
SENSPESTI	Tissue engineering of living biosensors to evaluate risks for health by pesticides affecting the cholinergic neurotranmitter system
SAFE FOODS	Promoting food safety through a new integrated risk analysis approach for foods
SYSTEQ	The development, validation and implementation of human systemic toxic equivalencies (TEQs) as biomarkers for dioxin-like compounds
TESTMETEDECO	Development of test methods for the detection and characterisation of endocrine disrupting chemicals in environmental species

European Environment Agency

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The Weybridge+15 (1996–2011) report

2012 — 112 pp. — 21 x 29.7 cm

ISBN 978-92-9213-307-8
doi:10.2800/41462

TH-AK-12-002-EN-N
doi:10.2800/41462

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ISBN 978-92-9213-307-8

European Environment Agency