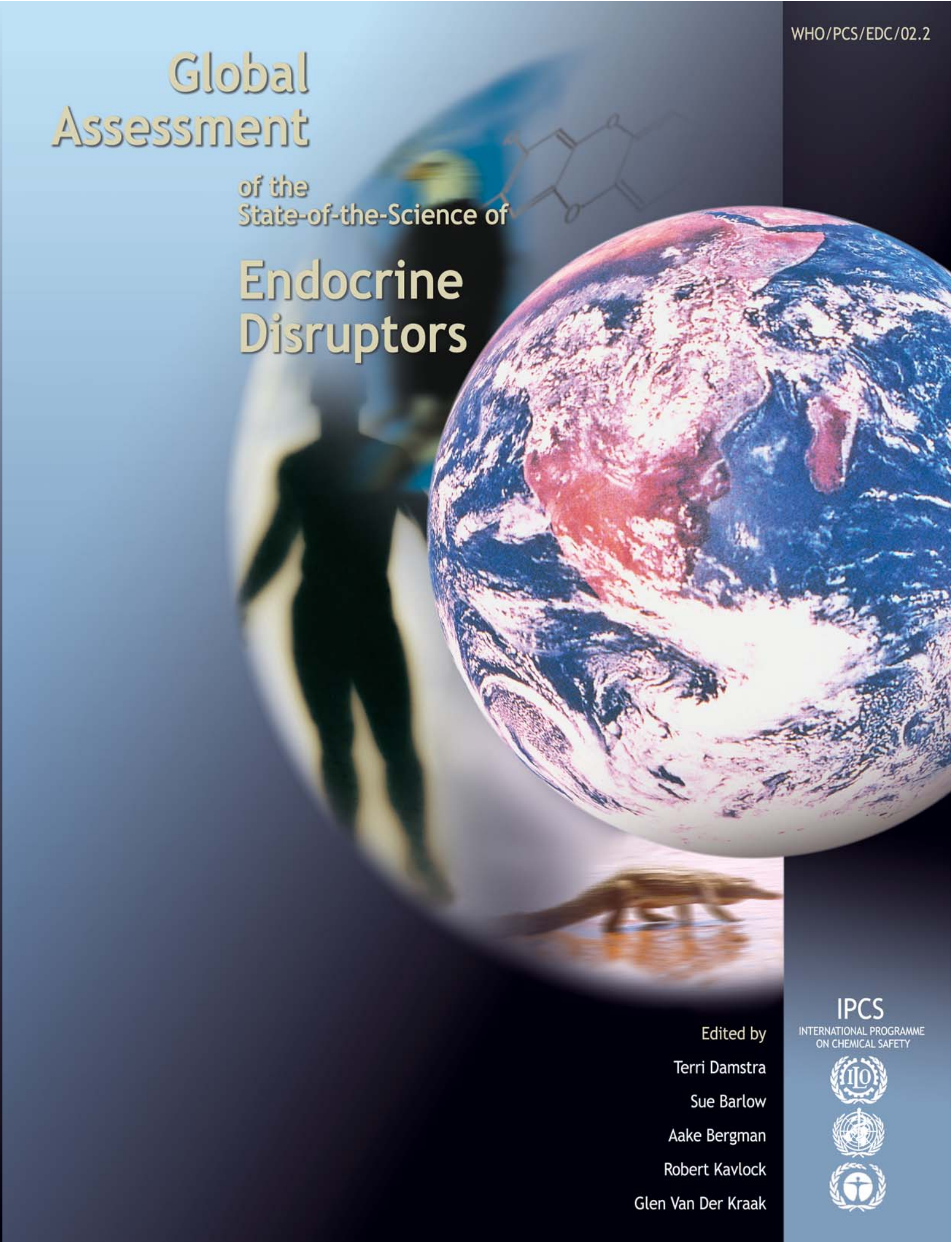
WHO/PCS/EDC/02.2

Global Assessment

of the
State-of-the-Science of

Endocrine Disruptors



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IPCS
INTERNATIONAL PROGRAMME
ON CHEMICAL SAFETY

International Programme on Chemical Safety

GLOBAL ASSESSMENT OF THE STATE-OF-THE-SCIENCE OF ENDOCRINE DISRUPTORS

An assessment prepared by an expert group on behalf of the World Health Organization, the International Labour Organisation, and the United Nations Environment Programme

Edited by: Terri Damstra, Sue Barlow, Aake Bergman, Robert Kavlock, Glen Van Der Kraak

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The International Programme on Chemical Safety (IPCS), established in 1980, is a joint venture of the United Nations Environment Programme (UNEP), the International Labour Organisation (ILO), and the World Health Organization (WHO). The overall objectives of the IPCS are to establish the scientific basis for assessing the risk to human health and the environment from exposure to chemicals, through international peer-review processes, as a prerequisite for the promotion of chemical safety, and to provide technical assistance in strengthening national capacities for the sound management of chemicals.

The Inter-Organization Programme for the Sound Management of Chemicals (IOMC) was established in 1995 by UNEP, ILO, the Food and Agriculture Organization of the United Nations, WHO, the United Nations Industrial Development Organization, the United Nations Institute for Training and Research, and the Organisation for Economic Co-operation and Development (Participating Organizations), following recommendations made by the 1992 United Nations Conference on Environment and Development to strengthen cooperation and increase coordination in the field of chemical safety. The purpose of the IOMC is to promote coordination of the policies and activities pursued by the Participating Organizations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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Contents

Preface

Global concerns have been raised in recent years over the potential adverse effects that may result from exposure to chemicals that have the potential to interfere with the endocrine system. Wildlife and human health effects of EDCs were first proclaimed by Rachel Carson in 1962, and based on a growing body of knowledge, those concerns have increased. This concern regarding EDCs is directed at both humans and wildlife. In response to these concerns, the Second Session (February 1997) of the Intergovernmental Forum on Chemical Safety made a number of recommendations to the Member Organizations of the **IOMC**, notably, IPCS and OECD, concerning approaches and means for coordinating and/or supporting efforts to address the issues internationally, including the development of an international inventory of research and coordinated testing and assessment strategies. This endorsed earlier recommendations from an international workshop at the Smithsonian (January 1997) and was followed by the 1997 Declaration of the Environmental Leaders of the Eight on Children's Environmental Health, which specifically addressed the issue of EDCs in their declaration. The environment leaders encouraged continuing efforts to compile an international inventory of research activities, develop an international assessment of the state of the science, identify and prioritize research needs and data gaps, and develop a mechanism for coordinating and cooperating on filing of the research needs. The 50th World Health Assembly adopted resolution WHO 50.13 in 1997, which called upon the Director-General of WHO to "take the necessary steps to reinforce WHO leadership in undertaking risk assessment as a basis for tackling high priority problems as they emerge, and in promoting and coordinating related research, for example, on potential endocrine-related health effects of exposure to chemicals."

List of Abbreviations

EDCs Endocrine-disrupting chemicals
ILO International Labour Organization
IOMC Inter-Organization Programme for the Sound Management of Chemicals
IPCS International Programme on Chemical Safety
OECD Organisation for Economic Co-operation and Development
UNEP United Nations Environment Programme
USA United States of America
US EPA United States Environmental Protection Agency
WHO World Health Organization

In response to these recommendations, the International Programme on Chemical Safety (PCS) of the WHO/UNEP/ILO assumed responsibility for developing this global assessment of the current state of scientific knowledge relative to environmental endocrine disruption. Concurrently, the IPCS assisted in the development of a Global Endocrine Disruptor Research Inventory (see *http://endocrine.ei.jrc.it*), which serves as a tool to foster complementary research efforts and identify strengths and weaknesses of current global research efforts.

The IPCS (in collaboration with the OECD) convened an informal consultation in 1997 and a Scoping Meeting in 1998 to outline the objectives, scope, and development process for the assessment document. The IPCS established a Steering Group of the following scientific experts to provide oversight, expertise, and guidance for the project and to evaluate the accuracy, significance, and relevance of the information in the document.

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This Steering Group met seven times over a three-year period to evaluate and revise various drafts of the document. Several of the Steering Group members served as chapter coordinators and editors and provided significant text contributions. Their continuing commitment to this document was essential for its completion.

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Without their significant text contribution, this task could not have been completed and the IPCS is extremely grateful.

A preliminary draft of the document was circulated to numerous scientific experts and IPCS contact points for their review. The IPCS offers special thanks to the following individuals for their substantial review comments:

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Dr Terri Damstra, IPCS, was responsible for the preparation of the final document. Editorial assistance was provided by Ms Kathleen Cameron (London, United Kingdom) and Dr Larry Fishbein (Fairfax, Virginia), and Ms Kathy Prout, IPCS (Research Triangle Park, North Carolina) provided word processing assistance.

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Chapter 1: Executive Summary

1.1 Purpose and Scope of Document

The last two decades have witnessed growing scientific concerns and public debate over the potential adverse effects that may result from exposure to a group of chemicals that have the potential to alter the normal functioning of the endocrine system in wildlife and humans. Concerns regarding exposure to these EDCs are due primarily to 1) adverse effects observed in certain wildlife, fish, and ecosystems; 2) the increased incidence of certain endocrine-related human diseases; and 3) endocrine disruption resulting from exposure to certain environmental chemicals observed in laboratory experimental animals. These concerns have stimulated many national governments, international organizations, scientific societies, the chemical industry, and public interest groups to establish research programs, organize conferences and workshops, and form expert groups and committees to address and evaluate EDC-related issues. Many of the proceedings of these workshops and/or committees have been published (see Table 2.1) and served as background material for this publication.

However, in the light of continuing uncertainties and highly publicized concerns, the International Programme on Chemical Safety was requested to provide an objective, global assessment of the current state-of-the-science relative to environmental endocrine disruption in humans, experimental studies, and wildlife species. This assessment builds on existing reviews and documents but is not intended to 1) cover all of the endocrine systems that may be disrupted by environmental exposures, 2) assess available test methodologies for detecting EDCs, or 3) address risk assessment and risk management issues. Rather, it focuses on the global peer-reviewed scientific literature where the associations between environmental exposures and adverse outcomes have been demonstrated or hypothesized to occur via mechanisms of endocrine disruption. Endocrine disruption is not considered a toxicological end point per se but a functional change that may lead to adverse effects. For the purposes of this document, a slight modification of the Weybridge (1996) definition was used and endocrine disruptors are defined in a generic sense as follows:

> An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.
>
> A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations.

List of Abbreviations

DDE Dichlorodiphenyl dichloroethylene
DDT Dichlorodiphenyl trichloroethane
EDCs Endocrine-disrupting chemicals
GLEMEDS Great Lakes embryo mortality, edema, and deformity syndrome
PCBs Polychlorinated biphenyls
TBT Tributyl tin

Concerns regarding EDCs have generated a vast number of divergent research studies conducted under various conditions and examining various outcomes. It is extremely rare that a single study could provide all the necessary relevant information to link a particular exposure scenario to a particular health outcome in wildlife or humans. Therefore, it is essential to evaluate the entire body of relevant knowledge. A unique feature of this assessment document for evaluating diverse data sets is that it provides a framework and utilizes objective criteria for assessing causality between exposures to EDCs and selected outcomes (see Chapter 7).

Chapter 2 summarizes critical generic issues (e.g., exposure–outcome associations, dose–response relationships, role of natural hormones and phytoestrogens, etc.), several of which are particularly relevant to EDCs.

Chapter 3 provides background information on the endocrine system, the role of hormones, and potential mechanisms of endocrine disruption along with specific chemical examples of multiple modes of action. The emphasis is on the vertebrate endocrine system and on the hypothalamic-pituitary-gonad, hypothalamic-pituitary-adrenal, and hypothalamic-pituitary-thyroid axes.

Potential adverse outcomes in both wildlife (Chapter 4) and humans (Chapter 5) have focused mainly on reproductive and sexual development and function; altered immune, nervous system, and thyroid function; and hormone-related cancers. Selected data sets illustrating exposure to certain EDCs in different parts of the world are discussed in Chapter 6, along with a discussion of exposure issues particularly relevant to EDCs.

As mentioned, Chapter 7 describes a framework for evaluating the collective information from diverse data sets in a structured manner to provide objective assessments of the state-of-the-science of determining causality between exposures to EDCs and selected outcomes. Chapter 8 summarizes the conclusions and lists some general research recommendations.

1.2 Endocrine Mechanisms of Action

Research has clearly shown that EDCs can act at multiple sites via multiple mechanisms of action. Receptor-mediated mechanisms have received the most attention, but other mechanisms (e.g., hormone synthesis, transport, and metabolism) have been shown to be equally important. For most associations reported between exposure to EDCs and a variety of biologic outcomes, the mechanism(s) of action are poorly understood. This makes it difficult to distinguish between direct and indirect effects and primary versus secondary effects of exposure to EDCs. It also indicates that considerable caution is necessary in extrapolating from *in vitro* data to *in vivo* effects, in predicting effects from limited *in vivo* data, and in extrapolating from experimental data to the human situation. A collective weight of evidence is essential in determining under what conditions observed effects resulting from exposure to EDCs occur via endocrine-mediated mechanisms. This document outlines a number of criteria that can be used as a basis for attribution of an effect to an endocrine-mediated mechanism (see section 3.16).

Despite an overall lack of knowledge of mechanisms of action of EDCs, there are several examples where the mechanism of action is clearly related to direct perturbations of endocrine function and ultimately to adverse *in vivo* effects (see section 3.12). These examples also illustrate the following important issues:

- Exposure to EDCs during the period when "programming" of the endocrine system is in progress may result in a permanent change of function or sensitivity to stimulatory/inhibitory signals.
- Exposure in adulthood may be compensated for by normal homeostatic mechanisms and may therefore not result in any significant or detectable effects.
- Exposure to the same level of an endocrine signal during different life history stages or during different seasons may produce different effects.
- Because of cross talk between different components of the endocrine systems, effects may occur unpredictably in endocrine target tissues other than the system predicted to be affected.

Considerable data are available on the early molecular events involved in hormone response, but there is little knowledge of the relationship between these molecular events and the potential for adverse health outcomes. Until such data become available, it will remain difficult and controversial to attribute adverse effects due to endocrine-mediated pathways.

1.3 Dose–Response Relationships

The issue of dose–response relationships is perhaps the most controversial issue regarding EDCs. One of the reasons is that EDCs often act by mimicking or antagonizing the actions of naturally occurring hormones. These hormones (often more potent than exogenous EDCs) are present at physiologically functional concentrations, so the dose–response considerations for EDCs are often different than for other environmental chemicals, which are not acting directly on the endocrine system. Reports of low-dose effects of EDCs are highly controversial and the subject of intense research. Dose–response relationships are likely to vary for different chemicals and endocrine mechanisms. Timing of exposure is absolutely critical to the understanding of dose–response relationships for EDCs. This is true for both wildlife and humans and for cancer as well as for developmental, reproductive, immunological, and neurological effects. Numerous examples exist in the literature where age at exposure is a known risk factor.

1.4 Effects in Wildlife

Several field and laboratory studies have shown that exposure to certain EDCs has contributed to adverse effects in some wildlife species and populations. These effects vary from subtle changes in the physiology and sexual behavior of species to permanently altered sexual differentiation. Most of the data come from Europe and North America. Aquatic species (at the top of the food chain) are most affected, but effects have also been observed in terrestrial species. Some adverse effects observed in certain species are likely to be endocrine mediated, but in most cases, the causal link between exposure and endocrine disruption is unclear. Examples include the following:

Mammals: Exposure to organochlorines (PCBs, DDE) has been shown to adversely impact the reproductive and immune function in Baltic seals, resulting in marked population declines. These seals exhibit a compromised endocrine system, but precise mechanisms of action remain unclear.

Birds: Eggshell thinning and altered gonadal development have been observed in birds of prey exposed to DDT, resulting in severe population declines. A syndrome of embryonic abnormalities (known as GLEMEDS) has been observed in fish-eating birds and can be directly related to PCB exposure, but the precise linkage to endocrine function is uncertain.

Reptiles: A presumed pesticide spill in Lake Apopka (Florida, USA) provides a well-publicized example of potential EDC effects on population decline in alligators. A variety of gonadal and developmental abnormalities were observed that have been attributed to high levels of various organochlorine contaminants that disrupt endocrine homeostasis. Several hypotheses have been proposed to explain the contaminant-induced endocrine disruption, but the precise cause(s) is not known.

Amphibians: Population declines in amphibians has been observed in both pristine and polluted habitats worldwide. Currently, the data are insufficient to implicate EDCs as causative agents.

Fish: There is extensive evidence that chemical constituents present in pulp and paper mill effluents and sewage treatment effluents can affect reproductive endocrine function and contribute to alteration in reproductive development. A variety of mechanisms (e.g., hormone–receptor interactions, interference with sex steroid biosynthesis, altered pituitary function) are involved, but precise modes of action or the causative chemicals are still poorly understood.

Invertebrates: Exposure of marine gastropods to TBT (a biocide used in antifouling paints) provides the clearest example in invertebrates of an endocrine-mediated adverse effect caused by exposure to an environmental contaminant. Masculinization of marine gastropods exposed to TBT has resulted in worldwide declines of gastropods. The endocrine mechanism probably involves elevated androgen levels possibly through altered aromatase activity.

Studies in wildlife have been proposed as "sentinels" of human exposure to EDCs. However, given the diversity of wildlife, caution must be taken in extrapolating the responses to EDCs, as research has focused primarily on only a few species of wildlife. Also, potential effects of EDCs on wildlife tend to focus on the individual, whereas ecological risk assessments focus on populations and communities. The significance of disturbances in reproductive output and viability of offspring on populations is difficult to quantify. Overall, the current scientific knowledge provides evidence that certain effects observed in wildlife can be attributed to chemicals that function as EDCs. However, in most cases, the evidence of a causal link is weak, and most effects have been observed in areas where chemical contamination is high.

1.5 Human Health Effects

Analysis of the human data by itself, while generating concerns, has so far failed to provide firm evidence of direct causal associations between low-level (i.e., levels measured in the general population) exposure to chemicals with EDCs and adverse health outcomes. It is difficult to compare and integrate results from diverse human studies, because data are often collected at different time periods, using different experimental designs and under different exposure conditions. Often exposure data are completely lacking. Of particular concern is the lack of exposure data during critical periods of development that influence later functioning in adult life. Furthermore, the concentrations and potencies of endogenous hormones and phytoestrogens are generally higher than those of exogenous chemicals. Despite these difficulties, exposure to EDCs has been suggested to play a role in adverse health outcomes, and concerns remain. The following examples illustrate these concerns:

Reproductive Effects: A number of studies report a decline (since the 1930s) in human sperm quality in several countries. There clearly are important variations in sperm count, both within and between countries, but there are no firm data that directly addressed

the possible cause and effect relationship between declining sperm quality and exposure to EDCs. Studies to date have been retrospective. Several meta-analyses of existing studies reached different conclusions, and the issue remains controversial. Even if there has been deterioration in semen quality, this would not necessarily be due to endocrine disruption.

Available human and experimental animal studies demonstrate that high-level exposure to certain environmental chemicals can impair fertility and increase the rate of spontaneous abortion, but the relationship to endocrine disruption remains speculative.

Declining sex ratios (fewer males) have been recorded in a number of regions and countries, and there is evidence that unidentified external influences are associated with such changes, but the mechanism(s) is unknown.

Temporal increases in the frequency of development abnormalities of the male reproductive tract, particularly cryptorchidism and hypospadias, have been reported, but the role of exposure to EDCs is unclear. Experimental data show that a number of chemicals can disrupt development of the male reproductive tract via endocrine mechanisms.

Endometriosis: Exposure to certain EDCs has been reported to be associated with endometriosis, but the studies remain equivocal.

Precocious Puberty: Concerns have been raised about the influence of EDCs on the timing of puberty, but the possible mechanisms of action and role of other factors such as nutrition need to be clarified.

Neural Function: Data from human and experimental animal studies clearly indicate that exposure (particularly prenatal exposure) to certain EDCs (e.g., PCBs) can have adverse effects on neurological development, neuroendocrine function, and behavior. Some of these effects appear to result from altered thyroid or neurotransmitter function, but in most instances endocrine mechanisms have not been demonstrated. Similar effects can also result from exposure to chemicals that induce developmental neurotoxicity but have no known endocrine action.

Immune Function: Exposure to environmental chemicals, including certain EDCs, has been shown to alter immune function in humans and animals. However, it is not clear whether such impaired function is due to endocrine-mediated mechanisms.

Cancer: Temporal increases in the incidence of certain cancers listed below in hormonally sensitive tissues in many parts of the industrialized world are often cited as evidence that widespread exposure of the general population to EDCs has had adverse impacts on human health. These increases cannot be adequately explained by improved diagnostic techniques, and it has been argued that these trends coincide roughly with the increased use and release of industrial chemicals into the environment.

Breast Cancer: Numerous human epidemiological studies and experimental laboratory studies have been conducted to determine whether environmental EDCs may contribute to an increased risk of breast cancer, but the current scientific evidence does not support a direct association between exposure to environmental EDCs and increased risk of breast cancer. However, studies published to date have measured EDC exposure levels in adult women; data on exposures during critical periods of development are lacking. Adult women currently at risk for breast cancer may have been exposed to exogenous EDCs *in utero* or during infancy, childhood, and adolescence in the mid-twentieth century when contaminant levels of organochlorines were higher.

Endometrial Cancer: Limited available data do not support a causative role for EDCs in endometrial cancer.

Testicular Cancer: Temporal increases in the incidence of testicular cancer have been reported in certain countries, but rates vary considerably among countries. The risk started rising around 1910 in Nordic countries, and somewhat earlier in England and Wales, and therefore cannot be attributed solely to chemicals introduced in the mid or late twentieth century. Some evidence suggests that the incidence of cryptorchidism and hypospadias may show similar geographic variations to the incidence of testicular cancer and that these conditions may be developmentally linked. However, EDC exposure data for critical periods are lacking.

Prostate Cancer: Exposure to certain pesticides and organochlorines has been linked to increases in the incidence of prostate cancer in a few limited studies, but most studies have found no association, and the mechanism is unknown.

Thyroid Cancer: A direct association between exposure to EDCs and thyroid cancer has not been demonstrated.

Overall, the biological plausibility of possible damage to certain human functions (particularly reproductive and developing systems) from exposure to EDCs seems strong when viewed against the background of known influences of endogenous and exogenous hormones on many of these processes. Furthermore, the evidence of adverse outcomes in wildlife and laboratory animals exposed to EDCs substantiates human concerns. The changes in human health trends in some areas (for some outcomes) are also sufficient to warrant concern and make this area a high research priority, but non-EDC mechanisms also need to be explored.

1.6 Exposure

Often the weakest link in determining whether observed adverse effects in humans and/or wildlife are linked to EDCs is the absence of adequate exposure data. Often data are limited to accidentally highly exposed groups. Most exposure information has focused on the presence of persistent organic pollutants in Europe and North America. Data on the magnitude and trends of global human or wildlife exposure are limited. Potential sources of exposure are through contaminated food, contaminated groundwater, combustion sources, and contaminants in consumer products. Information on exposure during critical development periods is generally lacking. The exposure data sets that exist are primarily for various environmental media (air, food, water) rather than the most relevant internal exposure (blood, tissue). Limited exceptions are human breast milk and adipose tissue samples. Worldwide, despite large expenditures of money, time, and effort, comparable data sets for assessing exposures to EDCs for humans or wildlife are not available. Such information is essential to adequately evaluate exposure–response relationships in field and epidemiology studies and to use these relationships to produce credible risk assessments.

1.7 Causal Criteria and Weight of Evidence for Effects Resulting from Exposure to EDCs

Chapter 7 outlines a structured format [based on modifications of criteria proposed by Bradford-Hill (1965), Fox et al. (1991), and Ankley et al. (1997)] for assessing postulated relationships between altered health outcomes and exposure to EDCs. Examples (see Tables 7.1 and 7.2) were selected to illustrate the broad range of data (or lack thereof) available for determining the overall strength of the evidence for causal associations for a particular outcome and exposure of concern. These examples illustrate that for many hypotheses there are insufficient data to reach any definitive conclusions. However, in some examples there is sufficient evidence for endocrine-mediated effects to warrant concerns.

2.1 General Background

Since the publication of Rachel Carson's *Silent Spring* (Carson, 1962), there has been increasing awareness that chemicals in the environment can exert profound and deleterious effects on wildlife populations and that human health is inextricably linked to the health of the environment. The last two decades, in particular, have witnessed a growing scientific concern, public debate, and media attention over the possible deleterious effects in humans and wildlife that may result from exposure to chemicals that have the potential to interfere with the endocrine system. The intensity of the concerns and lack of consensus among scientists can best be ameliorated by an objective evaluation of the available scientific data on the potential adverse effects of these chemicals from a global perspective. Countries lacking the necessary infrastructure to monitor and evaluate these chemicals expressed a particular need for an objective international assessment. The document builds on existing assessment documents and reviews (see Table 2.1) and is not intended as a thorough, comprehensive literature review. Only peer-reviewed literature or publicly available reports were evaluated. It is **not** a risk assessment or consensus document. Neither is it an assessment of available test methodologies for detecting EDCs. Both the OECD and a number of national organizations are addressing these issues (ECETOC, 1996; OECD, 1998a, 1998b, 1999a; US EPA, 1998a; Kanno et al., 2000).

EDCs encompass a variety of chemical classes, including natural and synthetic hormones, plant constituents, pesticides, compounds used in the plastics industry and in consumer products, and other industrial by-products and pollutants. They are often pervasive and widely dispersed in the environment. Some are persistent, can be transported long distances across national boundaries, and have been found in virtually all regions of the world. Others are rapidly degraded in the environment or human body or may be present for only short periods of time but at critical periods of development.

List of Abbreviations

AhR Aryl hydrocarbon receptor
DDE Dichlorodiphenyl dichloroethylene
DDT Dichlorodiphenyl trichloroethane
EDCs Endocrine-disrupting chemicals
ECETOC European Centre for Ecotoxicology and Toxicology of Chemicals
ER Estrogen receptor (α and β isoforms)
IUPAC International Union of Pure and Applied Chemistry
JEA Japan Environment Agency
LH Luteinizing hormone
MRC Medical Research Council
NRC National Research Council
NTP National Toxicology Program
OECD Organisation for Economic Co-operation and Development
PCBs Polychlorinated biphenyls
PCDDs Polychlorinated dibenzodioxins
PCDFs Polychlorinated dibenzofurans
POPs Persistent organic pollutants
SETAC Society for Environmental Toxicology and Chemistry
TCDD 2,3,7,8-Tetrachlorodibenzyl-*p*-dioxin
UBA Umweltbundesamt (German Environmental Agency)
UNEP United Nations Environment Programme
US EPA United States Environmental Protection Agency

2.2 Generic Issues

There are a number of complex issues that must be considered when evaluating the effects of endocrine disruptors (Ashby et al., 1997b; Ashby, 2000). These are summarized in this chapter and are discussed in detail in subsequent chapters (detailed references are also provided in subsequent chapters). Studies that clearly address exposure–outcome relationships are the most valuable in assessing the impact of EDCs on wildlife and human health. Unfortunately, many of the epidemiological or wildlife studies do not have good measures of exposure, which limits our ability to draw firm conclusions from them. This problem is especially prevalent for those EDCs that are rapidly degraded in the environment or in the human body. This means that the exposure that might have caused an adverse outcome (e.g., a reproductive deficit) is not detectable at the time that clinical manifestations become evident. For this reason, most of the EDCs that are relied on to draw cause-and-effect relationships are those that are biologically and ecologically persistent (e.g., PCBs, DDT, and dioxins). A number of these POPs are known to endanger human health and ecosystems and have been the subject of global conventions. Twelve POPs were singled out for elimination and/or reduction in a legally binding global treaty, which was signed by 115 countries in Stockholm in May 2001. These initial 12 high-priority POPs were selected based on data that demonstrate adverse exposure–outcome relationships in humans and wildlife, and processes are being implemented to add additional chemicals to the list.

This document emphasizes those chemical exposures and adverse human and ecological outcomes where an indication of cause and effect via multiple mechanisms of endocrine disruption has been demonstrated or hypothesized. These case studies also illustrate the types of interferences that are of importance and the variety of adverse health outcomes that can be exhibited.

One of the major issues that needs to be addressed when evaluating the impact of EDCs on human health and the environment is whether effects reported in the literature represent isolated cases or more global responses. For example, diminished wildlife populations adjacent to a significant point source may not be indicative of global responses. In contrast, relatively small effects on wildlife or human health points might have great impact if those responses are global in nature. Another problem in assessing the health impacts of EDCs is that some of these chemicals have been shown to contribute to the incidence of common diseases of multifactorial etiology (e.g., infertility, cancer, neurobehavioral deficits). Therefore, it will be difficult to attribute effects in traditional epidemiology studies to EDCs unless those effects are seen in large numbers of people.

2.3 Mechanisms of Endocrine Disruption in Humans and Wildlife

There are a number of mechanisms whereby EDCs can modulate endocrine systems and potentially cause adverse effects (see also Chapter 3). The generally accepted paradigm for receptor-mediated responses includes binding of hormone to its receptor at the cell surface, cytoplasm, or nucleus, followed by a complex series of events that lead to changes in gene expression characteristic for a specific hormone (Birnbaum, 1994). It is thought that changes in gene expression represent an early but critical step in the regulation

Table 2.1 - Selected Workshop/Committee/Assessment Reports on Endocrine Disruption

Year	Organization	Purpose/Scope	Reference
1992	World Wildlife Fund	Examination of the commonalities of adverse effects in wildlife, experimental animals, and humans. Produced the "Wingspread Consensus Statement"	Colborn and Clement, 1992
1994	National Institute of Environmental Health Sciences	Review of cellular biology, developmental effects, sources, and global health implications of environmental estrogens	Maclachlan and Korach, 1995
1995	German Federal Environmental Agency	Discussion about the occurrence and impact of endocrine disruptors and the potential risks that may arise to humans and the environment	German Federal Environment Agency, 1996
1995	Ministry of Environment and Energy, Denmark	Evaluation of the effects of estrogens on male reproductive development and function	Toppari et al., 1996
1995	US EPA	Workshop on research needs for the Risk Assessment of health and environmental effects of endocrine disruptors (April 1995)	Kavlock et al., 1996
1995	Chemical Manufacturer's Association; World Wildlife Fund; US EPA	Workshop on screening methods for chemicals that alter thyroid hormone homeostasis, action, and function	Ankley et al., 1998a
1995	UK Medical Research Council Institute for Environment & Health	Assessment on Environmental Estrogens: Consequences to Human Health and Wildlife	MRC Institute for Environment and Health, 1995
1996	European Commission	European workshop on the impact of endocrine disruptors on human health and wildlife, Wyebridge U.K.	European Commission, 1996
1996	ECETOC	Compendium of test methods for environmental estrogens	ECETOC, 1996
1996	SETAC	Workshop on principles and processes for evaluating endocrine disruption in wildlife	Kendall et al., 1998
1996	US Committee on the Environment and Natural Resources	Development of a national planning framework for endocrine disruptor research and analysis of the existing federally funded research projects to help identify information gaps	Reiter et al., 1998
1996	US EPA	Workshop on the development of a risk strategy for assessing the ecological risk of endocrine disruption	Ankley et al., 1997
1997	UNEP, US EPA, White House Office of Science and Technology; Alton Jones Foundation	International workshop on endocrine disruptors	UNEP, 1997
1997	Federal Environment Agency, Germany	Workshop on effects of endocrine disruptors on neuronal development and behavior	UBA, 1997
1997	SETAC, OECD, European Commission	Expert Workshop on Endocrine Modulators and Wildlife: Assessment and Testing	SETAC, 1997
1997	US EPA	Special report on environmental endocrine disruption: an effects assessment and analysis	US EPA, 1997
1997	OECD	Critical assessment of the ability of existing OECD test methods to detect sex hormones disrupting potential	OECD, 1997
1997	Health Council of the Netherlands	Evaluation of the effects of endocrine disruptors on human reproduction and development	Health Council, Netherlands, 1997
1997	International Life Sciences Institute; US EPA	Scientific evaluation of the potential for substances in the diet to influence the human endocrine system	ILSI, 1998
1997	Japan Chemical Industry Association	Evaluation of status and research needs of endocrine disrupting compounds in Japan	Japan Chemical Industry Association, 1997
1998	Swedish Environmental Protection Agency	Endocrine disrupting substances-impairment of reproduction and development	Olsson et al., 1998
1998	International Union of Pharmacology	Natural and anthropogenic environmental estrogens—the scientific basis for risk assessment	IUPAC, 1998
1998	Society of Environmental Toxicology and Chemistry	Workshop on endocrine disruption in invertebrates	DeFur et al., 1999
1999	National Research Council	Hormonally active agents in the environment	NRC, 1999
1999	European Commission	Scientific committee on toxicity, ecotoxicity, and the environment opinion on human and wildlife health effects of endocrine disruptors	Vos et al., 2000
2000	Health and Environment Canada	Workshop on endocrine disrupting substances in the Canadian environment	Servos and Van Der Kraak, 2001
2000	US National Toxicology Program	Report of the endocrine disruptors low-dose peer review	NTP, 2001a
2000	Finnish Environment Institute	Research for Management of Environmental Risks from Endocrine Disruptors	Assmuth and Louekari, 2000
2001	Federal Environment Agency, Germany	Second status seminar on endocrine disruptors	UBA, 2001a

of normal biological function, including cell proliferation and differentiation responses essential for normal development and the function of multiple organ systems. Although there is considerable information on the early molecular events involved in hormone response, there is very little knowledge concerning the relationship between those molecular events and adverse health effects such as cancer or reproductive toxicity. This knowledge gap is perhaps the most limiting factor in our ability to evaluate exposure–response relationships, particularly following low-level exposure to potential EDCs. The use of new approaches in molecular epidemiology and animal model systems has the potential to yield additional valuable information for elucidating the role of these mechanistic determinants of specificity at low-dose exposures to potential EDCs and for improved risk evaluations for the adverse health effects of EDCs. There are numerous experimental systems available for evaluating the interactions of exogenous or synthetic chemicals with hormone systems, particularly those that interact with the estrogen, androgen, thyroid, and AhRs (Bolander, 1994). However, there is a growing body of knowledge that interactions of chemicals with other receptor systems also may be important in the EDC arena. These include the retinoic acid receptor, cytokine systems, and a number of so-called orphan receptors (receptors without unknown ligands and/or functions) such as the peroxisome proliferator receptor system. In general, these receptor systems are remarkably well conserved phylogenetically, suggesting that data from wildlife and experimental models should be useful, although not necessarily definitive, for estimating risks from EDC exposure to humans.

The mechanism or mode of action of EDCs is not limited to those agents that interact directly with hormone receptors. Other mechanisms of interest include inhibition of hormone synthesis, transport, or metabolism and activation of receptor through processors such as receptor phosphorylation or the release of cellular complexes necessary for hormone action. In the case of hormone synthesis, considerable research has been conducted on the aromatase inhibitors that prevent the conversion of androgens to estrogens through a cytochrome P450 system that is highly conserved in many species. Several fungicides have been shown to cause infertility by aromatase inhibition. In addition, there is a growing awareness that multiple receptor systems act in concert to regulate biological functions. For example, "cross talk" between the ER and growth factor receptors appears necessary for estrogen signaling of mammary cells to divide or differentiate. These events are critical for explaining several risk factors for breast cancer such as age at menarche, age at menopause, or effects of numbers of pregnancies. There are numerous other kinds of cross talk between various constituents of the endocrine system, and an understanding of the mechanisms involved could improve our ability to produce credible health assessments of EDCs. One well-known example of "cross talk" involves antiandrogen-mediated elevation of endogenous estrogen levels due to increased LH production.

Other examples of EDCs capable of acting at multiple cellular sites via multiple endocrine mechanisms are discussed in detail in Chapter 3. For example, the pesticide methoxychlor displays estrogen agonist (ER-β) activity because some of its metabolites bind to the ER. It also possesses antiandrogenic actions through a more poorly defined mechanism involving the hypothalamic-pituitary-gonadal axis. Another example is the ability of the DDT metabolite DDE to act as an antiandrogen by inhibiting testosterone binding with the androgen receptor, but these antiandrogenic effects may also be facilitated by the effects of DDE on steroid-metabolizing enzyme expression.

There are many factors that should be considered when utilizing mechanistic information on EDCs in health assessments. Of particular concern are species, interindividual, and tissue specificity in endocrine signaling pathways. Differential responsiveness to EDCs has been observed between different species and extends to interindividual differences within a species and between different tissues as well. The biologic and molecular mechanisms underlying this specificity are quite diverse. Determinants of species specificity include differences that exist between species in receptor binding, gene transcription patterns of gene expression, and cellular responses to endocrine-active compounds. Interindividual differences in responsiveness may be determined at the level of genetic polymorphisms in hormone-metabolizing enzymes, hormone receptors, and those genes that are activated by these receptors. Our rapidly growing knowledge base emerging from the human genome project will enable the design of rational studies on the impact of EDC exposures on hormonally sensitive end points in groups that may be genetically predisposed. Extrinsic factors such as diet can also impact individual susceptibility to endocrine-active agents.

The organochlorine chemicals provide interesting insights regarding the mechanisms of action for EDCs. In the case of the dioxins, the scientific consensus is that most, if not all, of their effects require an initial interaction with a cellular protein termed AhR (Poland and Glover, 1977). As discussed in Chapter 3, the ligand-bound AhR is capable of interacting with a number of critical signal transduction pathways, leading, for example, to involvement with xenobiotic metabolizing enzymes, steroid receptor signaling, growth factor expression, circadian clocks, responses to hypoxia, and angiogenesis. Through these varied interactions, such chemicals are able to induce a wide spectrum of biological effects at a number of different life stages in a variety of species, and some of these responses do not easily fit the traditional definition of endocrine-mediated events. Although adverse biological effects mediated via the activation of AhR are certainly of concern, a higher burden of proof is needed to identify such effects as due to endocrine disruption than merely the correlation between ability to bind to the receptor and the elicitation of some biological effect. In this regard, the considerations detailed in section 3.16 were used in evaluating the extent to which information would be included in this document. Although information on the involvement of the AhR in wildlife is limited, in our final conclusions on the strength of the evidence for relationships between chemicals and endocrine-mediated effects, the same criteria were applied for both humans and wildlife.

2.4 Dose–Response Relationships

The issue of dose–response relationships is perhaps the most controversial issue regarding EDCs. One of the reasons is that EDCs often act by mimicking or antagonizing the actions of naturally occurring hormones. These hormones are already at physiologically functional concentrations, so the dose–response considerations for EDCs are often different than for other chemicals that are not acting directly on the endocrine system. Reported low-dose effects for EDCs have come under intense scrutiny regarding the question of the adequacy of traditional toxicology testing paradigms for detecting low-dose effects. A recent workshop on this issue (NTP, 2001a) concluded that although low-dose effects may be occurring, those effects often are not replicated

consistently, and the toxicological significance of the reported effects is not known. Dose–response issues should be explicitly considered when studies are designed for risk evaluation for health or wildlife effects. Of particular relevance is the issue of dose selection. Ideally, the doses used should span a wide range to identify both toxic and mechanistic end points. The issue of dose selection has become critical to the current controversies surrounding the issue of biphasic dose–response curves for EDC effects on end points such as prostate weight. Although there may never be complete knowledge on the mechanism(s) of action for any chemical, some knowledge of key events could help clarify dose–response relationships.

Timing of exposure is also critical to the understanding of dose–response relationships for EDCs. Numerous examples exist in the literature where age at exposure is a known risk factor. For example, endocrine disruption of the developing brain can permanently alter behavior, whereas similar exposures to a fully differentiated brain could be without effect. Ecological and wildlife effects are also strongly influenced by the timing of exposure (e.g., during the breeding season).

Population heterogeneity is another important factor in dose–response evaluation. For human health, a number of factors contribute to a wide range of risks, including genetic predisposition, age, gender, diet, disease conditions, and past exposures. The range of risk modulators may be even greater for complex ecosystems, but little information is available in this area.

Evaluation of the dose–response relationships for health and environmental effects of endocrine disruptors will be most credible when information is available from several sources (e.g., toxicity studies, mechanistic and epidemiological studies, and field studies). There are a number of issues that are helpful to consider when embarking on dose–response assessment. These include, but are not limited to, 1) the adequacy of relevant experimental models for evaluating potential human effects of low-dose exposure to endocrine disruptors, 2) state of knowledge concerning quantitative relationships among the various processes maintaining homeostasis for the tissue, organ, or function being studied, 3) how perturbations in homeostasis lead to disease or dysfunction, 4) whether these changes can be quantified, 5) an understanding of the mechanisms through which endocrine disruptors perturb homeostasis and endocrine function and alter the risks from that of normal levels of endogenous hormones, 6) consideration of how differences in lifestyle factors (diet, nutrition, etc.) affect sensitivity to endocrine disruption, 7) an understanding of how the age of the endocrine system alters sensitivity to endocrine disruption, and 8) how interindividual differences (based on genetic variation) in constituents of endocrine pathways (e.g., receptor variants) alter responses to endocrine-sensitive end points caused by exposure to EDCs. Most of these considerations are relevant to both human and wildlife effects of EDCs.

A common dose–response relationship for all effects and for all endocrine disruption mechanisms should not be expected. This conclusion is based on the knowledge that there are many different kinds of hormonal actions of chemicals categorized as endocrine disruptors. These activities include estrogenic, antiestrogenic, antiandrogenic, growth factor modulation, cytokine and thyroid modulation, modulation of hormone metabolism, among many others.

2.5 Exposure Issues

There are numerous chemicals in the environment (e.g., pesticides, industrial chemicals, and natural products) that are hormonally active, and these can be detected in people and wildlife as well as in environmental samples. Some of these persist in the environment and others do not. Some are lipophilic, sequestered in adipose tissue and secreted in milk, and others may only be present for short periods of time but at critical periods of development. Our knowledge about the magnitude of human or wildlife exposure remains very limited. Most of the more definitive studies on chemically mediated effects, including those on EDCs, have been conducted on highly exposed groups in various occupations or from accidental exposures. In only a few cases, appropriate exposure information is available from lower level environmental exposures because of analytical sensitivity and the latency in outcome after exposure has occurred.

Hormonally active environmental chemicals are extraordinarily diverse in their structure and potency. For example, some organohalogens, such as the PCBs, DDT, PCDDs, and PCDFs, are suspected endocrine-disrupting agents, but various members of these groups of chemicals exhibit profound differences in potency, biological and ecological persistence, and mechanisms of action. For example, 75 PCDD congeners and 135 PCDF congeners vary tremendously in their potency to exhibit TCDD-like activity (see Chapter 6). This kind of diversity creates obvious problems in human and ecological health assessments, and it also increases the complexity and costs of analyzing for concentrations of these chemicals in biological samples. In addition to the PCBs, PCDDs, and PCDFs, many other kinds of chemical modulators of endocrine function are examined in this report. These include phthalate acid esters, DDT and DDE, alkylphenols, methoxychlor, bisphenol, diethylstilbestrol, estradiol as an ecological contaminant, the fungicide vinclozolin, and several other synthetic chemicals that are reported to interact with various components of the endocrine system.

Synthetic chemicals are not the only exogenous agents that have caused health concerns because of their hormonelike activity. Of particular interest are the phytoestrogens (such as genistein and equol) and the fungal estrogens (such as zearalenone). The phytoestrogens and fungal estrogens are diverse in structure, undergo complex metabolic processes, and are ubiquitous in the environment. They can be found in blood and urine samples of virtually every person and animal on this planet, often in high concentrations. They pose difficult analytical issues, yet if exposure–response relationships for the phytoestrogens remain uncertain, health assessments for many endocrine-disrupting agents, particularly the environmental estrogens, will also remain uncertain. This is because several of the phytoestrogens, mostly notably genistein and its analogs, possess binding affinities for the ER far greater than many of the EDCs of concern, such as the alkylphenols, bisphenol A, and DDE. From a potency standpoint, the phytoestrogens exert a far greater impact on human exposure to exogenous estrogens than do the synthetic chemicals. This does not mean that we should not be concerned about synthetic estrogens, but it does emphasize that exposure assessments for EDCs need to consider both the magnitude of exposure and relative potencies of the array of EDCs that may be encountered in the home, workplace, and general environment.

Information is needed to more accurately quantify the human, wildlife, and environmental burden of hormonally active environmental chemicals so that quantitative comparisons can be made between body levels of natural and exogenous hormones based on potency, not just absolute amount. This kind of information is essential if we ever hope to properly evaluate

exposure–response relationships in field and epidemiology studies and to use those relationships to produce credible risk assessments. Data on historical and geographic trends of exposure to EDCs are generally lacking. Knowledge of the fate and transport of new and existing chemicals is also limited, particularly among the different environmental compartments (water, sediment, and biota).

Exposure assessment, particularly as it involves human health, must focus on vulnerable groups, in terms of both life stage and lifestyle. Exposure assessment for the critical development stages remains a high research priority. These stages include gestation, lactation, adolescence, and senescence. The endocrine system, through a developmentally regulated pattern of expression, controls the pathways essential for cell proliferation, differentiation, and organ development, so it is not surprising that perturbations of the endocrine system during critical windows of sensitivity create the greatest potential for adverse health effects.

Vulnerability of different groups in the population will be affected by lifestyle factors (e.g., subsistence hunting and fishing, and avid sportsmen who consume fish and wildlife), genetic factors (e.g., metabolic differences that can determine sensitivity), special dietary habits, and age (e.g., the types and rates of food consumption in children). Although there is general agreement that diet would likely be the major exposure route for exposure to the EDCs, an approach based on integrated exposure assessment needs to be taken. All routes should be examined (e.g., dermal, inhalation, and ingestion). Examining the exposure of humans or wildlife to multiple chemicals (especially for chemicals with a common mode of action and/or common target sites) that may function as EDCs is also critical.

Exposure assessment encompasses both external measurements (levels in air, water, soil, food, etc.) and internal measurements (levels in blood, urine, and tissue samples). Both kinds of measurements provide critical information for wildlife, epidemiological, and experimental studies. Internal measurements are often confounded by the rapid metabolism of some EDCs (Elsby et al., 2001). This means that quantification of metabolites or degradation products in biological samples is necessary for endocrine disruptor research. Some of the rapidly metabolized chemicals reviewed in this document are the phthalate acid esters, alkylphenols, diethylstilbestrol, some PCBs, phytoestrogens, and methoxychlor.

Other complications in exposure assessment include time lags, seasonality, and multiple chemical exposures. a) Time lags between exposure and effect: The transgenerational nature of some EDC effects may be the single most complicating factor. All of the potential latent effects that may occur from short-term exposures during critical development windows have not yet been identified. b) Seasonality: Because of the sensitivity of reproductive stages to EDCs, seasonality will be extremely important to wildlife. In addition, the association of EDCs with the aquatic environment is complicated by seasonal rainfall, storm runoff, and water releases. c) Multiple chemical exposures: This, too, is a factor for any toxic chemical, but it is especially identified here because of the potential for effect modification (e.g., synergy, additivity, or antagonism).

The most critical need on status and trends is for the continuation and improvement of monitoring of the environment for the presence and magnitude of contaminants. Although environmental and tissue levels of certain EDCs (e.g., PCBs) have declined in some countries in response to regulations, they remain of concern in other countries, and uncertainty still exists regarding future trends. For most EDCs, data on trends are not available. Long-term data using harmonized collection and analysis methods are needed. Existing programs that furnish repeated measures of chemical contamination in the environment or in food provide our only indication of whether exposure is increasing or decreasing, and to what magnitude.

3.1 Introduction to Endocrine Systems

Endocrine systems of the body play an essential and pervasive role in both the short-and long-term regulation of metabolic processes. Nutritional, behavioral, and reproductive processes are intricately regulated by endocrine systems, as are growth (including bone growth/remodeling), gut, cardiovascular, and kidney function and responses to all forms of stress. Disorders of any of the endocrine systems, involving both overactive and underactive hormone secretion, result inevitably in disease, the effects of which may extend to many different organs and functions and are often debilitating or life-threatening. Viewed from this general perspective, the threat posed from environmental chemicals with endocrine activity (either agonist or antagonistic) is potentially serious. However, the fact that humans and wildlife are exposed to such chemicals does not necessarily mean that clinically manifest disturbance of the relevant endocrine system will result, because much depends on the level and duration of exposure and on the timing of exposure.

3.2 Scope and Terminology

3.2.1 Overview

The endocrine system originally was considered to consist only of glands that secreted hormones into the blood that traveled to distant target tissues, bound to specific cellular receptors, and produced characteristic actions. Currently, our concept of "endocrine" has been broadened by the discovery of other chemical regulators, such as chemicals secreted into the blood by neurons, that are sometimes termed neurohormones. The term "cytocrine" has been applied to numerous local or intercellular chemical regulators, including growth factors. Intercellular cytocrines that travel through the extracellular fluids to other cells in a tissue also are known as paracrine and autocrine regulators, depending on whether they affect other cells or themselves, respectively. The term "intracrine" has been suggested for intracellular regulators such as second messengers and transcription factors. Even before allowing for the increase in complexity of "endocrinology" that has resulted from recent recognition of the many cytocrine/paracrine systems that operate, it had been realized that there were numerous "classical" endocrine systems in the body that regulate processes as diverse as blood pressure, smooth muscle contraction, fluid balance, and bone resorption.

It is beyond the scope of this chapter to describe the entire endocrine system; instead, the focus will be on the three major endocrine axes that affect reproductive development and function. This restriction is based on the observations that many manifestations of endocrine disruption involve the reproductive system, particularly during its vulnerable developmental period. The particular aspects of the endocrine system that are covered include the HPG, the HPT, and the HPA axes. This restriction is arbitrary and should not imply that endocrine disruptors cannot affect other endocrine axes. It is also emphasized that the general principles on which all endocrine (and probably paracrine) axes are first set up and then operate are essentially identical, and hence, most of what is discussed below can be transferred in principle to other endocrine axes that are not described. The emphasis will be on the vertebrate endocrine system, with only minor attention paid to invertebrates. Although there are many parallels between vertebrate and invertebrate endocrine mechanisms, there are some major differences as well. General discussions of invertebrate endocrinology have been reported (Downer and Laufer, 1983; Matsumoto and Ishii, 1997; Cymborowski, 1992; Nijhout, 1994). This chapter consists of two main parts: sections 3.1–3.11 detail the normal functioning of the endocrine system, both in adults and in the developing organism; sections 3.12–3.16 focus on the impact of endocrine disruptors on organ systems and disease processes. The largest of the sections deals with effects on reproductive system development using several well-characterized examples from the experimental literature (e.g., MXC, vinclozolin, ketaconazole,

List of Abbreviations

17α,20β-P 17α,20β-dihydroxy-4-pregnen-3-one
5-HT Serotonin
ACTH Adrenocorticotropin hormone
AGD Anogenital distance
AhR Aryl hydrocarbon receptor
AR Androgen receptor
ARNT AhR nuclear translocator
AFP α-Fetoprotein
BBP Butylbenzyl phthalate
BNF β-Napthoflavone
cAMP Cyclic AMP
CBG Corticotropin-binding globulin
CRH Corticotropin-releasing hormone
CYP Cytochrome P
DBP Di-*n*-butyl phthalate
DDE Dichlorodiphenyl dichloroethylene
DDT Dichlorodiphenyl trichloroethane
DEHP Di-ethylhexyl phthalate
DEP Diethyl phthalate
DES Diethylstilbestrol
DHEA Dihydroepiandrosterone
DHP Dihexyl phthalate
DHT Dihydrotestosterone
DMP Dimethyl phthalate
DOTP Dioctyl phthalate
E_2 17β-Estradiol
EDCs Endocrine-disrupting chemicals
ER Estrogen receptor (α and β isoforms)
FSH Follicle-stimulating hormone
Gal_4-HEGO Gal_4-human estrogen receptor construct
GH Growth hormone
GnRH Gonadotropin-releasing hormone
GSI Gonadal-somatic index
GTH Gonadotropin (isoforms I and II)
HIF-1α Hypoxia inducible factor 1α
HPA Hypothalamic-pituitary-adrenal
HPG Hypothalamic-pituitary-gonadal
HPOA Hypothalamic preoptic area
HPT Hypothalamic-pituitary-thyroid
HPTE 2,2-Bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane
IL Interleukin
IUGR Intrauterine growth retardation
LE Long-Evans
LH Luteinizing hormone
LOAEL Lowest observed adverse effect level
M1, M2 Vinclozolin metabolites
MEHP Mono-ethylhexyl phthalate
MIH Müllerian inhibiting hormone
MIS Anti-Müllerian substance
mRNA Messenger RNA
MXC Methoxychlor
NOAEL No observed adverse effect level
PCBs Polychlorinated biphenyls
PCDFs Polychlorinated dibenzofurans
PGs Prostaglandins
PRL Prolactin
SARMs Selective androgen receptor modulators
SD Sprague-Dawley
SERMs Selective estrogen receptor modulators
SHBG Sex hormone–binding globulin
T_3 Triiodothyronine
T_4 Thyroxine
TCDD 2,3,7,8-Tetrachlorodibenzyl-*p*-dioxin
TRH Thyrotropin-releasing hormone
TSH Thyroid-stimulating hormone
US EPA United States Environmental Protection Agency

phthalates, and dioxin). These examples were selected to provide a broad view of the basic modes of action that are involved in the interaction of chemicals with the endocrine system. In addition to describing the modes of actions, descriptions of the critical periods, dose sensitivity, and resulting phenotypes seen in experimental models are provided. Similarly to the section on normal endocrine function, this section deals primarily with effects on vertebrates, and mammals in particular. Succeeding sections provide examples of EDC-related modes of action pertinent to carcinogenesis and the function of the nervous and immune systems. The final section provides a overall framework to judge whether a particular outcome, whether observed in the laboratory, in the field, or in an epidemiology setting, could be related to an EDC-related mode of action. This framework is intended to provide a structure by which subsequent observations, either contained in this assessment or reported subsequently in the scientific literature, can be judged relative to ascertainment of the mode of action.

3.2.2 Homeostasis

The fundamental role of all endocrine systems is to enable a dynamic, coordinated response of a distant target tissue to signals originating from another organ and, in some instances, cues originating from outside of the body. For most endocrine systems, the primary objective is to maintain some form of "homeostasis," avoiding wild swings in hormone levels/responses that might otherwise have detrimental metabolic effects (Norman and Litwack, 1998). A good example is the role of insulin in maintaining blood glucose levels within the normal range, that is, a range that does not fall so low as to result in unconsciousness and does not rise so high that wasteful excretion/spillage into urine occurs. When insulin levels do not respond to changing blood levels of glucose, diseases such as diabetes are the result. All endocrine systems operate to a large extent on the "seesaw" principle (Figure 3.1), in which the target cells send feedback signals (usually negative feedback) to the regulating cells, with the result that secretion of the target cell–stimulating hormone is altered (usually reduced) by one or more of the products of the target cells (Darlington and Dallman, 1995). However, in reality, there are usually elaborations or refinements of this simple archetypal endocrine system that enable all of the endocrine systems of the body to be integrated via cross talk. The reasons for this are obvious. For example, reproduction needs to take account of age, nutritional status, and in most animals, season of the year. Similarly, stress responses, and to a lesser extent, endocrine systems regulating hunger, need to be able to override other endocrine systems when danger threatens. This cross talk is vital for a healthy life and has important implications for the evaluation of endocrine disruptors. Exposure to an estrogenic chemical, for example, may affect not only the reproductive endocrine axis but also several other endocrine systems as well as bone, fat, and cardiovascular systems.

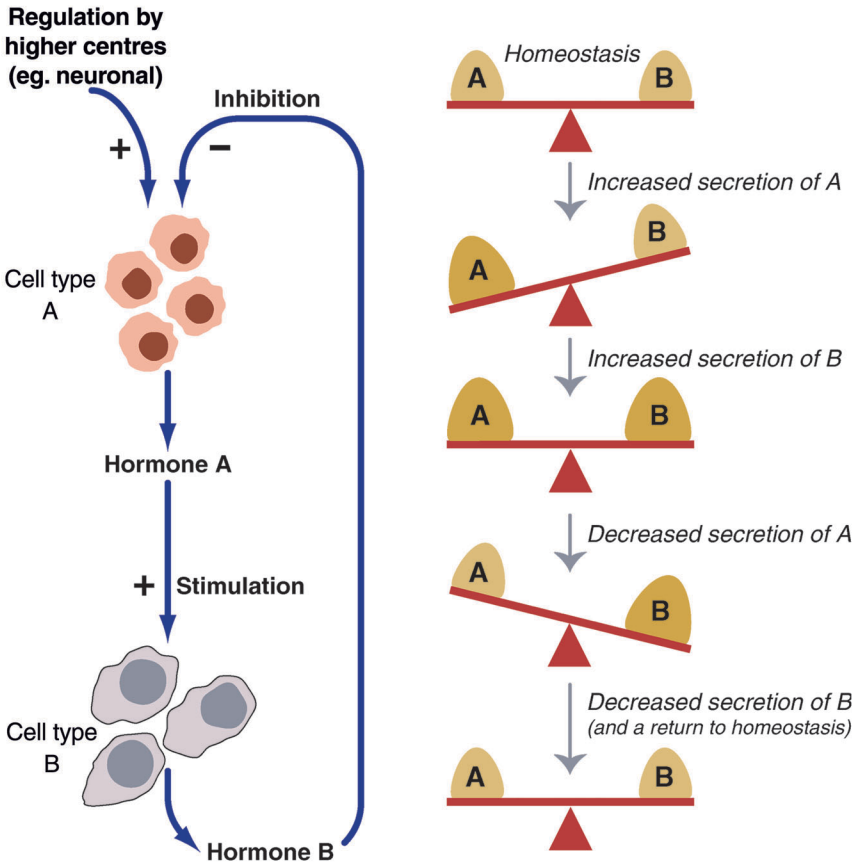


Figure 3.1 - Schematic diagram illustrating the basic "seesaw" principle on which endocrine systems work. Cell type A secretes hormone A, which regulates production of hormone B by cell type B, and in turn, hormone B exerts negative feedback regulation of the secretion of hormone A. In this way, swings in secretion of hormone A or B will be compensated for to maintain homeostasis (i.e., the correct levels of A and B), as shown on the right. This general principle operates in most, if not all, endocrine and paracrine systems, although in reality there are usually additional factors that will interplay in the regulation of levels of A and B.

3.2.3 Programming of Endocrine Axes

Although homeostasis, via seesaw-type mechanisms, is a central feature of all endocrine systems, it should be stressed that the balance between the two sides of the "seesaw" need to be set up or programmed before the system will work correctly. This programming will determine at what level the two sides of the seesaw will begin to respond to signals from the other side (Figure 3.1). For many of the endocrine systems, it appears that the setup program is established during fetal/neonatal development in mammals and that an abnormal environment at this stage of life can result in permanent misprogramming (De Kloet et al., 1988; Seckl, 1999). A good example of this is what happens as a result of fetal IUGR. Although such offspring often reach normal growth postnatally, they show a high incidence of insulin resistance (higher than normal insulin levels) and, consequently, are at increased risk of diabetes, obesity, and cardiovascular disease in later life; they are also prone to precocious puberty. These changes are believed to represent an adaptation of the fetus to its suboptimal nutritional supply and may result from elevation of glucocorticoid levels in the fetus (Philips et al., 1998). A more specific example concerns programming of the hypothalamus of the female, but not of the

male, to respond to gradually rising estrogen levels by triggering a positive response—the ovulatory GnRH-driven LH surge. In mammals, this programming is established perinatally, and exposure of the female at this time to moderate levels of male sex steroids will prevent this programming and render the female permanently infertile because of anovulation (Dohler, 1991). In contrast, exposure of the adult female to the same male sex steroids will not alter this programming, although it may temporarily disrupt ovulation by increasing negative feedback (Figure 3.2).

3.2.4 Impact of Endocrine Disruptors

In considering the potential impact of endocrine disruptors on bodily functions, the following points are critical :

(1) Exposure in adulthood may be compensated for by normal homeostatic mechanisms and may therefore not result in any significant or detectable effect.
(2) Exposure during the period when programming of the endocrine system is in progress may result in a permanent change of function or sensitivity to stimulatory/inhibitory signals.
(3) Exposure to the same level of an endocrine signal at different stages in the life history or in different seasons may produce different effects.
(4) Because of cross talk between different endocrine systems, effects may occur unpredictably in endocrine systems other than the system predicted to be affected. This is true for each of the situations in (1) through (3) above.
(5) In view of (4), considerable caution should be exercised in extrapolating *in vitro* measures of hormonal activity to the situation *in vivo*.

3.3 The HPG Axis in Mammals

3.3.1 Overview of the HPG Axis

This axis (Figure 3.2) involves three component parts: 1) GnRH neurons projecting from the hypothalamus of the brain; 2) gonadotropes in the anterior pituitary gland (adenohypophysis), which secrete the gonadotropins LH and FSH;, and 3) the somatic cells of the gonads (theca and granulosa cells in the ovary, Leydig and Sertoli cells in the testis). GnRH is secreted in pulses (Kimura and Funabashi, 1998; Terasawa, 1998) from the terminals of GnRH neurons and acts on the gonadotropes to induce secretion of both LH and FSH, which then act on their respective target cells in the gonads (LH on theca/Leydig cells; FSH on granulosa/Sertoli cells). Secretion of GnRH is modified by other neurons (e.g., Crowley, 1999), and the actions of GnRH on gonadotropin release may be modified by other hypothalamic or pituitary peptides (Evans, 1999). As a consequence, gonadal sex steroids stimulated by LH and the protein hormone inhibin (the A form in females, the B form in males) stimulated by FSH are released into the bloodstream and provide feedback to the hypothalamus and pituitary gonadotropes to reduce the secretion of GnRH, LH, and FSH, with inhibin selectively inhibiting FSH and the sex steroids inhibiting LH secretion (Crowley et al., 1991). This description implies that the arrangement of stimulatory and negative feedback loops complies with the simple arrangement shown in Figure 3.1. In reality, the arrangement is more complex and sophisticated. For example, the effects of GnRH on LH and FSH secretion are radically different, with LH release being stimulated very acutely (in

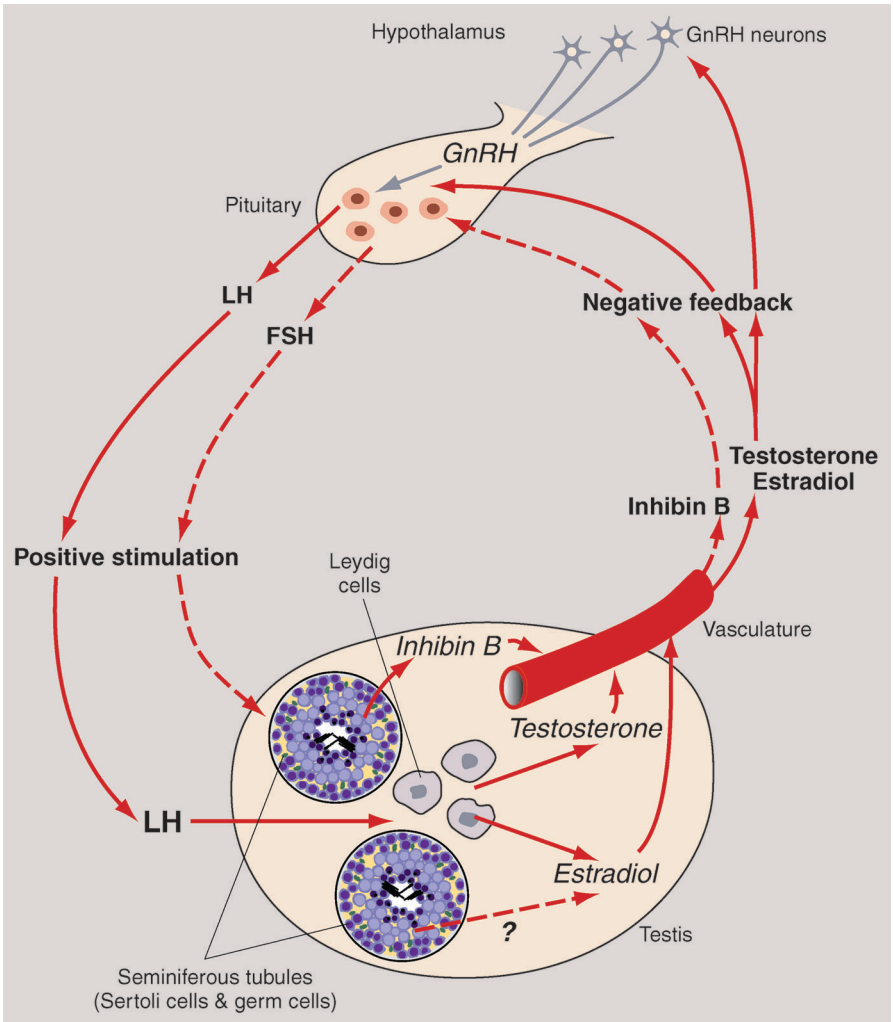


Figure 3.2 - Diagrammatic representation of the main working components of the mammalian HPG axis. The decapeptide GnRH is secreted from the terminals of GnRH neurons into the portal blood system, which delivers this "message" to gonadotrope cells in the anterior pituitary gland that express receptors for GnRH. Binding of GnRH to these receptors stimulates the synthesis and secretion into the bloodstream of the two gonadotropins LH and FSH. The gonadotropins then travel via the systemic bloodstream to reach their distant target cells in the gonad (a testis is shown for example). LH acts on Leydig cells to stimulate synthesis and secretion of testosterone, which in turn gains access to the bloodstream and via effects on the hypothalamus and anterior pituitary gland suppresses synthesis and secretion of GnRH and LH, respectively (= negative feedback). Similarly, FSH acts on Sertoli cells to drive the secretion of a protein hormone, inhibin B, which then travels via the bloodstream to the pituitary gland to suppress the synthesis and secretion of FSH (= negative feedback). Note that some of the negative feedback effects of testosterone may occur via its conversion to E_2, either in the testis (by Leydig cells and/or germ cells) or in the hypothalamus/pituitary gland. Note also that testosterone and/or E_2 exerts effects at many sites other than the hypothalamus and pituitary gland and that paracrine effects of these hormones, especially of testosterone within the testis, are also of vital importance. These and other refinements of the basic system illustrated here are outlined in the text.

pulses) by the GnRH pulses, whereas the response of FSH is extremely sluggish and takes many hours (Crowley et al., 1991; Bousfield et al., 1994). This stems from fundamental differences in GnRH-induced synthesis, packaging, and release of LH and FSH. Similarly, although the sex steroids (primarily testosterone in the male, E_2 in the female) negatively regulate LH secretion via effects on both GnRH secretion and gonadotrope function, they also exert some negative feedback on FSH secretion; in contrast, inhibin selectively inhibits FSH secretion.

3.3.2 Target Cell Sensitivity

In addition to these minor refinements of the archetypal endocrine system, there are other important factors that must be considered. One such factor is modulation of target cell sensitivity to stimulation. Gonadotropes do not exhibit a constant, unchanging response to GnRH stimulation, nor do the target cells in the gonads maintain unaltered responsiveness to LH/FSH stimulation. Sensitivity of the target cell to its stimulator is regulated both acutely and chronically (Conn, 1994; Erickson and Schreiber, 1995). For example, an abnormally high frequency of GnRH pulses or chronic exposure to GnRH or to agonistic analogues of GnRH (which are more resistant to degradation) results in loss or down-regulation of GnRH receptors on the gonadotropes, which serves to make them more resistant (= less sensitive) to further stimulation. This happens in a matter of hours and is followed more slowly by the gradual development of "desensitization" that involves changes to the GnRH-stimulated second messenger signaling mechanisms that reduce overall responsiveness of the gonadotropes to GnRH. Analogous mechanisms operate in gonadal cells to regulate their responsiveness to LH and, to a lesser extent, to FSH. In other words, each target cell in the endocrine axis also regulates its own responsiveness to stimulation. There is still further refinement of this process via cross talk between neighboring cells, especially in the gonads (Leung et al., 1992). There is good evidence, for example, that Sertoli cells in the testis are able to modulate both the numbers of LH receptors expressed in neighboring Leydig cells and their steroidogenic responsiveness via altering expression of steroid synthetic enzymes (Sharpe, 1993). In return, the testosterone secreted by Leydig cells exerts important paracrine regulatory effects on Sertoli cell function (Sharpe, 1994).

3.3.3 Metabolism of Endocrine Hormones

A further potentially modulable element in the component loops of the HPG axis is the metabolism of the secreted hormones. Increased or decreased catabolism, with a consequent change in half-life of a hormone, will change its effectiveness without altering its level of secretion. FSH has a naturally longer half-life than LH (i.e., it is metabolized more slowly), which is one reason why changes in FSH levels are more sluggish than changes in LH (Bousfield et al., 1994). Of much more importance is the role of proteins that bind the sex steroids. These include albumin and AFP in the fetus/neonate and, most important, SHBG in humans. Approximately 97–98% of testosterone and E_2 that circulate in blood in humans is bound to SHBG, and only 2–3% is free and thus biologically active (Moore and Bulbrook, 1988; Rosner, 1990). This arrangement has two important consequences: 1) the half-life of the sex steroids is considerably prolonged and 2) a new indirect pathway for regulating sex steroid action becomes evident: modulation of SHBG secretion (by the liver) can potentially alter levels of bioactive sex steroid without affecting any of the major component parts of the HPG axis. In practice, the main (stimulatory) regulators of SHBG production are the sex steroids themselves as well as other important regulators of SHBG production that are components of other endocrine systems (Moore and Bulbrook, 1988; Rosner, 1990). Similar arguments may apply to other binding proteins (e.g., AFP).

3.3.4 Interaction of Paracrine and Endocrine Components of the HPG Axis

Testosterone produced by Leydig cells acts on neighboring Sertoli cells (an example of a paracrine effect). This is arguably the most important role of testosterone in the male, as its effect on Sertoli cells is the main pathway via which spermatogenesis is supported (Sharpe, 1994). There are analogous effects in the ovary with androgens produced by the theca cells exerting paracrine effects on granulosa cells in the adjacent, developing follicle (Erickson and Schreiber, 1995). The most important consequence of the exposure of granulosa cells to testosterone is that they are then able to convert this androgen to E_2, which then exerts multiple endocrine effects in the uterus and elsewhere in the body, including its role in negative feedback. This conversion of testosterone to E_2 also occurs at many other sites in the body, in both the male and the female (Simpson et al., 1997; Sharpe, 1998). The ability of cells to express aromatase and/or 5α-reductase, and thus to transform an endocrine hormone (testosterone) into a locally acting paracrine hormone (E_2 or DHT; Figure 3.3), appears to be far more common (especially in the male) than was initially hypothesized. These sites of paracrine action obviously depend on the supply of substrate and thus on the main endocrine axis, and "leakage" of the paracrine-generated products into the general circulation may contribute to negative feedback, although conceptually this would not appear to be important. One or both of the component parts of the paracrine mechanisms illustrated in Figure 3.3 are now known to be expressed in bone, muscle, the cardiovascular system, adipose tissue, the pituitary gland, and brain as well as throughout the reproductive systems of both male and female (Simpson et al., 1997; Sharpe, 1998). Paracrine systems can be considered to act as local satellites of the major endocrine axis, their role being to serve local needs.

3.3.5 Developmental Role of the HPG Axis

As noted, the setting up of endocrine axes takes place largely during fetal/neonatal development. During this period, feedback sensitivity of the hypothalamus and pituitary gonadotropes to steroids from the gonads is established, and this will determine at what level of sex steroid a reduction in GnRH and/or LH/FSH secretion will be triggered. The details of how this occurs are incompletely understood but clearly involve programming of neuronal pathways (Dohler, 1991). At the same time, differences between male and female feedback centers are programmed (Dohler, 1991; Gorski, 1996). This is necessary because female reproduction usually operates around a reproductive cycle, for example, the estrous or menstrual cycle, whereas male reproduction is usually a continuous or more protracted, noncyclic event. Production of gonadal hormones in the female is therefore cyclic whereas in the male it is relatively uniform, apart from special periods such as puberty and seasonal infertility. Appropriate changes to the "wiring" of the hypothalamus of the male and female therefore have to be induced during development to ensure that the pituitary gland of an adult female will respond as in a female rather than as in a male. Testosterone produced during fetal/neonatal life plays a role in programming the development of a "male" hypothalamus and brain, and administration of testosterone to a female during this critical programming period will result in masculinization of hypothalamic

function and consequent acyclicity and anovulation in adulthood (Dohler, 1991; Gorski, 1996). The relative roles of testosterone, DHT, and E_2 vary from behavior to behavior in a species-specific manner. This is true for both organizational and activational influences. In some species, all three hormones play a role in masculinization (Cooke et al., 1998). Importantly, there are significant species differences in the organizational and activational control of the development of sexually dimorphic behaviors (Cooke et al., 1999). For example, in the rat, activation of malelike mounting behavior in the adult female rat does not require an organizational effect of hormones during prenatal life. In at least some strains of rats, this behavior can be activated by testosterone in adult females. In contrast to the rat, androgens play an important role in the organization of mounting behavior in the nonhuman primate (Goy, 1978; Pomerantz et al., 1985). This difference must be considered when extrapolating findings from the rat to humans. Rough-and-tumble play behavior is one of the few social behaviors that appears to be regulated by androgens in both the rat and rhesus monkey (Goy, 1978).

3.3.6 Role of Hormones in Mammalian Sex Differentiation

As well as leading to masculinization of the brain, perinatal testosterone secretion by the testis is also responsible for masculinization of the body in general. This involves the formation of male genitalia, but effects on the many organs throughout the body also occur at this time (Simpson and Rebar, 1995). The role of androgens in sex differentiation is well understood. Before sex differentiation, the mammalian embryo has the potential to develop a male or a female phenotype. Following gonadal sex differentiations, testicular sections induce differentiation of the male duct system and external genitalia. The development of phenotypic sex includes persistence of either the Wolffian (male) or Müllerian (female) duct system and differentiation of the external genitalia. Generally, masculinization of the tissue/organ in question results from local conversion of circulating testosterone to either DHT or E_2, as shown in Figure 3.3, but for some tissues, MIS is also involved.

The female avoids developing as a male by simply not switching on secretion of testosterone by the ovary, and it is largely the absence of this endocrine signal that results in phenotypic and endocrinotypic female development (Simpson and Rebar, 1995). The central role of testosterone in facilitating masculinization has two important downsides. First, if a genotypic male fails to make testosterone, it will not masculinize and will develop as a phenotypic female (but with testes). Second, and conversely, if a genotypic female is exposed to sufficient testosterone (or other androgens), she will be masculinized (but will have ovaries). There are numerous examples of both of these situations that result most commonly from inactivating mutations (e.g., in the AR, resulting in lack of masculinization in males) or from abnormal androgen production (usually by the adrenal glands) by the mother or female fetus that lead to masculinization of the developing female (Simpson and Rebar, 1995). It is also emphasized that these are not necessarily all-or-nothing events. Partial masculinization of the female or partial failure of masculinization of the male can occur, including potentially quite subtle effects. Where these affect the genitalia or other external phenotypic feature (e.g., presence/absence of horns), they may be easily detected, but if the effects are confined to the brain or to another organ, they may not be easily deducible.

Like the brain and the genitalia, other organ systems such as the liver and muscles are also "imprinted" by the hormonal milieu during development and hence may also be targets of xenobiotics that perturb the normal endocrine profile at various stages of life. For example, the development of the levator ani-bulbocavernosus muscles and their neural regulation has been employed as a model of organizational and activational roles of testosterone on the ontogeny of sexual dimorphisms in the rat (Breedlove et al., 1999). These tissues are also sexually dimorphic in humans, an effect whose critical period lies in the first trimester of pregnancy. The levator ani muscles and spinal nuclei of the bulbocavernosus are considerably larger in males than in female rats, a response that requires testosterone exposure during both the prenatal and postpubertal stages of life. As the levator ani lacks 5α-reductase, testosterone, and not DHT, is the hormone that initiates the malelike developmental pattern.

Perhaps the most important aspect of these various "programming" changes is their irreversibility. The greatest concern about environmental hormone disruptors is centered on the

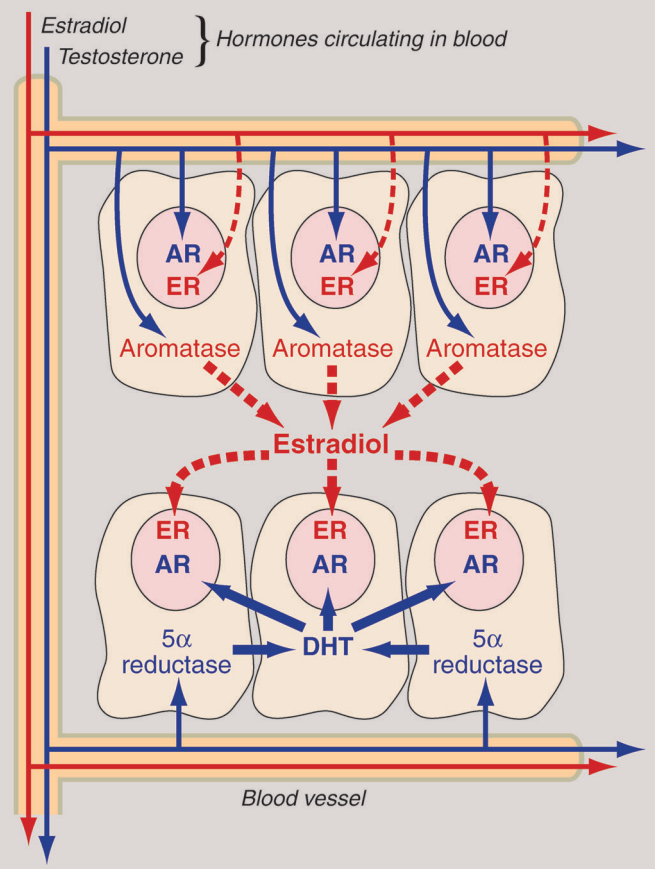


Figure 3.3 - Schematic illustration of the interplay between endocrine and paracrine regulatory systems as exemplified by androgens and estrogens. Testosterone and E_2 both circulate in the bloodstream as endocrine hormones, but within specific cell types testosterone can be converted to E_2 (via the enzyme aromatase), which can then interact with ERs or be converted to the more potent androgen, DHT, which can interact with ARs. Both of these conversion steps effectively amplify a relatively weak "endocrine" signal into a more powerful "paracrine" signal that can act locally on target cells. Because E_2 deriving from systemic and local sources will interact with the same ERs, and similarly, both testosterone from the systemic bloodstream and DHT derived locally will interact with the same ARs, this illustrates how endocrine and paracrine systems can interplay in a potentially powerful way using very simple mechanisms. The generation of locally high hormone levels to exert paracrine effects represents a means of regulating target cell function according to local needs of the tissue in question while still maintaining an overall endocrine influence on the same tissue via the blood-derived hormones.

possibility that exposure of an animal to such an agent during perinatal life can result in a permanent adverse or abnormal change; exposure to the hormone disruptor does not need to be chronic, as transient exposure at a critical time during development is all that is required. There is added uncertainty in this regard because of emerging understanding about how different androgens and estrogens may act in different tissues—so-called SERMs (Cosman and Lindsay, 1999) and corresponding SARMs (Negro-Vilar, 1999). The ability of such compounds to selectively activate or antagonize estrogen or androgen pathways in specific tissues presages the discovery of similar activities for certain environmental chemicals. Predicting the effects of such compounds in the context of programming of development of the reproductive system and its endocrine axis is extremely difficult.

3.3.7 The HPG Axis in Nonmammalian Species

Nonmammalian vertebrates differ greatly from mammals and one another in their modes of reproduction, with patterns of sequential and simultaneous hermaphroditism, parthenogenesis, viviparity, and gonochorism found in many major groups (van Tienhoven, 1983). Additionally, they may be more limited in their breeding frequencies. Some species breed only once (semelparous), whereas others may breed two or more times (iteroparous). The time of gonadal activity may be very short, with the gonads remaining quiescent during most of the year. Dissociated reproduction where testicular and ovarian development actually occurs at different times of the year also is known for numerous species (e.g., Houck and Woodley, 1994). However, the HPG axes of these animals are surprisingly similar in their operation, in the pattern of feedback mechanisms, and in the hormones involved to that described for mammals (for reviews, see Norris, 1997; Bentley, 1998).

GTH release is controlled in all nonmammals by a GnRH decapeptide molecule similar to that in mammals (Sherwood et al., 1994; Sower, 1998). As in mammals, these cells develop in the nasal placodes and migrate into the preoptic area and hypothalamus during early development (Dellovade et al., 1998). Typically, at least two forms of GnRH are found, but the second form (usually chicken GnRH-II, first isolated from chickens) functions primarily as a neurotransmitter or neuromodulator and probably influences reproductive behaviors rather than the HPG axis. Furthermore, many teleosts have three forms of GnRH present in the brain. A major difference occurs in the delivery of GnRH in teleostean fishes that lack a hypothalamo-hypophysial portal system between the hypothalamus and the pituitary and exhibit direct penetration of the adenohypophysis by GnRH axons. A portal delivery system also is lacking in the jawless fishes (agnathans), in which diffusion is the mode of delivery (Gorbman et al., 1999).

There are two distinct GTHs that are not directly homologous to mammalian FSH and LH. The first GTH, called GTH-I, is responsible for gonadal growth and gamete formation. The second, GTH-II, is involved with gamete release. Administration of sufficient amounts of either GTH can produce the effects of both, but they are secreted sequentially *in vivo*. Among the tetrapods (amphibians, reptiles, birds, and mammals), only the squamate reptiles (lizards, snakes) appear to have a single, FSH-like GTH, whereas all others produce both FSH-like and LH-like GTHs.

In general, testosterone is the major androgen produced in all vertebrates, and E_2 is the major estrogen. Many male teleosts also produce 11-ketotestosterone, and it is the predominant circulating androgen in many species. Female teleosts also produce testosterone, and circulating levels of testosterone may be as great as for E_2. Teleosts also produce important progesterone-like molecules, 17α,20β-P and 17,20β,21 trihydroxy-4-pregnen-3-one that cause final oocyte maturation and ovulation. It is secreted under the influence of GTH-II. This steroid may have pheromonal roles in mating as well. In some teleosts, the corticosteroid deoxycorticosterone produced by adrenocortical cells has been shown to induce final oocyte maturation and ovulation. Tetrapods secrete testosterone, E_2, and progesterone, which all play reproductive roles similar to those observed in mammalian development and reproduction. A secondary androgen secreted by all tetrapods is DHT. Female amphibians, like teleosts, exhibit high levels of androgens as well as estrogens during the reproductive portions of their life histories, but normally androgens are not prominent in the blood of female reptiles and birds. Knowledge of the mechanisms for steroid actions on target cells and characteristics of steroid appear to be similar to mammalian systems, although there are several differences. For example, in addition to ER-α and ER-β, a third distinct subtype, ER-γ, has been identified in teleosts (Hawkins et al., 2000). In addition, the teleost progestin receptor differs from its mammalian counterpart in its binding affinity for steroids and does not bind many EDCs that bind to the mammalian progesterone receptor (Pinter and Thomas, 1997). Thus, care must be taken in extrapolating the effects of EDCs across vertebrate taxa.

Complete or partial sex reversals of the gonads can be caused by early exposure of eggs, larvae, or juvenile animals to estrogens or androgens (Burns, 1961; Hayes, 1998a, 1998b), including many paradoxical effects such as feminization by androgens. Additionally, androgens usually inhibit female duct (Müllerian) development while enhancing male duct (Wolffian) development, whereas estrogens do the reverse. Androgens may enhance degeneration of the Müllerian ducts brought about by MIH or MIS. Estrogens are thought to protect oviducts from MIH (Norris, 1997), and paradoxical actions of androgens have also been reported (Norris et al., 1997a, 1997b; Clark et al., 1998). Clearly, endocrine disruptors that mimic estrogens or have antiandrogenic activity could potentially have drastic effects on wildlife exposed during development or as juveniles.

Estrogens can stimulate synthesis of ovalbumin protein by cells of the avian oviduct (Schlinger and Saldanha, 1998) and also the synthesis of vitellogenins by the liver (LeFleur, 1998; Meyer, 1999). Vitellogenins are precursors used by the ovaries to synthesize yolk proteins that are incorporated into eggs. The synthesis of vitellogenin (vitellogenesis) is a dramatic biomarker for estrogenic action in adult vertebrates that produce yolky eggs (fishes, amphibians, reptiles, and birds). Estrogens produce liver hyperplasia and hypertrophy as well as elevation of plasma vitellogenins. If males or immature females are exposed to estrogens, their livers also can be induced to produce vitellogenins (Matthiessen, 1998; Mattthiessen and Sumpter, 1998; LeFleur, 1998; Meyer, 1999). Thus, plasma vitellogenin can be used as a biomarker for exposure to environmental estrogens. In teleost fishes, amphibians, and birds, vitellogenesis is enhanced by PRL or GH, whereas in elasmobranchs, lizards, and turtles, PRL seems to play an inhibitory role (Grau and Weber, 1998). PRL also stimulates parental behaviors and may enhance estrogen-dependent secondary sex characters such as the avian brood patch (Jones, 1971).

Progesterone and progesteronelike hormones regulate non-genomic-based oocyte maturation in amphibians and teleost fishes, respectively (Paolucci et al., 1998), by binding to specific receptors on the cell surface. Oviductal secretion (Chester Jones et al., 1987) and the sensitivity of the amphibian oviduct to contract in the presence of arginine vasotocin (Guillette et al., 1985) are dependent

on progesterone. Progesterone apparently slows development of the young in the viviparous frog *Nectophrynoides ocidentalis* (Bentley, 1998). In turtles, progesterone decreases contractility as it does in mammals (Paolucci et al., 1998). The fact that steroids can induce rapid, cell-surface-mediated nongenomic actions by binding to specific receptors on the cell membrane has implications for the physiological processes they help regulate as well as their disruption by environmental chemicals (Revelli et al., 1998).

3.4 The HPA Axis

3.4.1 Overview of the HPA Axis

This axis operates in a similar way to that illustrated for the HPG axis, the major differences being in the regulatory and secretory molecules involved (Becker, 1995). CRH is secreted from the terminals of hypothalamic neurons and acts on corticotropes in the anterior pituitary gland to regulate the synthesis and secretion of ACTH, which is then transported via the bloodstream to the adrenal glands, where it stimulates the secretion of glucocorticoid hormones (cortisol and/or corticosterone). The latter have numerous effects throughout the body, including important roles in metabolism of carbohydrate, protein and fat, anti-inflammatory effects, and modulation of stress responses (Becker, 1995). As in other endocrine axes, the products of the target cell, the glucocorticoids, exert negative feedback effects at the hypothalamic and pituitary level to suppress CRH secretion. Similar to the sex steroid hormones, much of the glucocorticoid in circulation in blood is bound to a binding protein in the human (CBG), and local release of bioactive hormone from the CBG represents one mechanism of local tissue response to pro-inflammatory changes (Rosner, 1990). Increasingly, it is recognized that glucocorticoids have important "programming" effects during development and that alterations in the circulating levels of these hormones can affect the timing and set points of other endocrine axes. For example, the multiple consequences of IUGR, including the short- and long-term consequences in terms of disease risk, are believed to be triggered largely by elevation of glucocorticoid levels in the fetus (Philips et al., 1998).

Exposure to stresses or elevated glucocorticoid levels may have profound effects on brain development, as revealed by learning and memory deficits in adults. Early effects of handling newborn rats results in better regulatory control of the stress response as well as reduced cell losses in the hippocampus and less memory loss with aging (Francis et al., 1996; Meany et al., 1988). In contrast, elevated glucocorticoids in neonatal rats result in underdeveloped axonal growth as well as a reduction in myelination, formation of dendritic spines, and synaptogenesis, resulting in learning deficits and altered motor function (de Kloet et al., 1988).

There are a number of additional refinements/complexities to the HPA axis outlined above for mammals. Initially, the adrenal glands are a source of several other important hormones, including mineralocorticoids (which act on the kidney), opioid peptides, and enkephalins, as well as catecholamines, all of which have multiple effects throughout the body. Each of these secretions is regulated by mechanisms that are not related to the HPA axis. In the context of reproduction, the most important other products of the adrenal glands are the weak androgens DHEA, DHEA sulfate, and androstenedione, the secretion of which is also stimulated by ACTH. These adrenal androgens may be converted in target tissues to more potent androgens or to estrogens (Figure 3.3) and can therefore potentially affect functioning of the reproductive endocrine axis and the cell types that are responsive to androgens and estrogens (Simpson and Rebar, 1995). Overproduction of adrenal androgens can have major consequences, including partial sex reversal of the female fetus when this occurs *in utero* or in the pregnant mother (above). In the human, adrenal androgens also play a role in early puberty (adrenarche) and are responsible for stimulation of pubic and axillary hair growth (Ritzen, 1998). At the hypothalamic-pituitary level, additional control of ACTH release may be exercised via arginine vasopressin.

3.4.2 The HPA Axis in Nonmammals

Nonmammalian steroidogenic tissue homologous to the mammalian adrenal cortex may be termed the interrenal gland, interrenal tissue, or simply interrenal (Vinson et al., 1993; Norris, 1997a; Bentley, 1998). Hence, the HPA axis often is termed the hypothalamic-pituitary-interrenal axis, especially in fishes. This steroidogenic tissue is often referred to as "adrenocortical" to emphasize its homology to the adrenal cortex of mammals.

The HPA axis of nonmammals, like that of mammals, is stimulated by an initial secretion of a hypothalamic CRH-like peptide, followed in turn by release of ACTH from the pituitary and then secretion of cortisol (most fishes) or corticosterone (most amphibians, birds, and reptiles) from the adrenocortical tissue. The HPA axis is important in regulating responses to stress (Iwama et al., 1997) and appears to have anti-immune actions (Schreck, 1996) similar to those described in mammals (Gaillard, 1994). The organism may adapt to the presence of the stressor with the hormones returning to normal levels. Prolonged stress may be associated with an increase in the activity of the HPA axis, sometimes producing chronically elevated cortisol or corticosterone. In extreme cases, this may lead to exhaustion of the HPA axis and death. Chronically stressed animals may exhibit an activated HPA axis but present with normal or only slightly elevated level of glucocorticoids.

Although many nonmammals have been shown to secrete a mammalian-like CRH, other CRH-like peptides have been discovered in fishes and mammals that can increase ACTH secretion as well. Mammalian ACTH seems to be effective at stimulating adrenocortical secretion in all vertebrates, reflecting the conservation of amino acid sequence among the vertebrates in these peptides we call ACTH. A unique corticosteroid, 1-hydroxycorticosterone, is found among the elasmobranch fishes (sharks, skates, and rays), although they also produce corticosterone.

In teleostean fishes, cortisol functions both as a mineralocorticoid controlling Na^+ and K^+ balance and as a glucocorticoid. Small amounts of corticosterone also have been described in the plasma of teleosts. Larval and aquatic amphibians, like teleosts, produce cortisol as their major corticosteroid, but terrestrial and semiterrestrial amphibians secrete corticosterone, as do all reptiles and birds. Although all tetrapods produce aldosterone, its role in salt balance is not well studied in nonmammals. Mammals have two generalized corticosteroid receptors located in different target cells, respectively, for glucocorticoids and mineralocorticoids. The number of receptor types for corticosteroids in other vertebrates has not been examined intensively.

3.5 The HPT Axis

3.5.1 Overview of the HPT Axis

This axis operates in a very similar way to that illustrated for the HPG axis. TRH is secreted from the terminals of hypothalamic neurons and acts on thyrotropes in the anterior pituitary gland to

regulate the synthesis and secretion of TSH in mammals (Reed and Pangaro, 1995). TSH is then transported via the bloodstream to the thyroid gland, where it acts to stimulate the synthesis of T_3 and T_4, which are released into the bloodstream and act throughout the body to stimulate general metabolic activity. In practice, the main thyroid hormone released is T_4, although T_3 is biologically much more potent (Reed and Pangaro, 1995). In many target tissues, T_4 is metabolized to T_3, which then exerts its effects, another example of how a simple refinement to an endocrine axis can enable greater modulation and control according to local needs. Most of the circulating T_3 results from metabolic conversion of T_4 by a liver deiodinase. Circulating T_3/T_4 feeds back to the hypothalamus and anterior pituitary gland to negatively regulate TRH and TSH release, thus completing the classical endocrine negative feedback loop (Reed and Pangaro, 1995). Most of the feedback at the level of the pituitary gland is due to T_4 that is converted to T_3 in the thyrotrope. At the top end of this circuit, there is an additional controlling factor, somatostatin, which is released from hypothalamic neurons and exerts negative control of TSH release from the anterior pituitary gland. Somatostatin also plays a key role in the (negative) regulation of GH secretion from the anterior pituitary gland, an endocrine axis that is not discussed in this chapter. However, GH stimulates cell growth whereas TSH (via T_3/T_4) stimulates cell metabolism, showing that control of these two endocrine axes is interlinked at this level.

In the present context, interest in the thyroid endocrine axis stems from a) the demonstration that certain PCBs have antithyroidal activity, that is, can antagonize the effects of T_3/T_4 levels (Gray et al., 1993; Porterfield and Hendry, 1998); and b) the important role that the thyroid axis plays in terminal differentiation of various tissues, extending from neurons to muscle and to Sertoli cells in the testis. Many of the actions of thyroid hormones are permissive in that they affect the capacity of cells to respond to other stimuli. For example, the levels of the important enzyme adenyl cyclase, responsible for generation of the second-messenger cAMP in target cells for GH, is enhanced by thyroid hormones.

3.5.2 The HPT Axis in Nonmammals

The structure and functions of the HPT axis of nonmammals are very similar to those of mammals (McNabb, 1993; Norris, 1997b). The thyroid gland has a follicular arrangement, and the mechanisms of thyroid hormone synthesis and secretion as well as peripheral deiodination of T_4 to T_3 are very similar. Patterns of metabolic degradation and excretion also are similar. One major difference in bony fishes is the diffuse nature of the thyroid follicles that are not encapsulated by a connective tissue covering, such that individual follicles are distributed among the connective tissue elements between the second and fourth aortic arches. In some cases, thyroid follicles spread to other organs, including the kidney, liver, and gonads; hence, surgical thyroidectomy is not feasible in these animals.

The major differences in hypothalamic and pituitary regulation reside at the hypothalamic level. TRH is not the major stimulator of TSH release in fishes and amphibians. It appears that CRH is the principal releaser of TSH in amphibians (Denver, 1997). However, mammalian TSH is effective at stimulating iodide accumulation by the thyroid gland and secretion of thyroid hormones in all vertebrates. Thyroid hormones play critical roles in embryonic and postembryonic development of all vertebrates, especially as related to the nervous system. They are also important for the metamorphosis of larval fishes and amphibians into the juvenile body form (Dickhoff et al., 1990; Galton, 1992; Kikuyama et al., 1993; Shi, 1994). These actions not only bring about dramatic morphological changes but also involve biochemical adaptations related to marked changes in habitat and diets, that is, the roles of thyroid hormones and their interactions with PRL and corticosteroids in the smoltification of salmonid fishes and in amphibian metamorphosis. The parr-to-smolt transformation (smoltification) in juvenile salmonids fishes occurs prior to their seaward migration. Studies of coho salmon smoltification (Dickhoff et al., 1990) have documented two- to sixfold transient increases in T_4 and cortisol, respectively, during this time as well as early surges in insulin and PRL. GH also increases and levels remain elevated in smolts.

In larval amphibians, metamorphosis to the juvenile body form involves transient increases in thyroid hormones and corticosteroids as well as a late, brief rise in PRL. In addition, specific changes in the types of deiodinase enzymes result in decreasing conversion of T_4 to the inactive metabolite reverse T_3 and increases conversion of T_4 to the more active T_3 (Galton, 1992). Additionally, changes in thyroid hormone receptor types also occur during metamorphosis (Wolffe et al., 2000). Numerous gene products are up-regulated by thyroid hormones in responding tissues, and a few are down-regulated during metamorphosis (Shi, 1994).

Shedding of skin in salamanders and in reptiles is controlled by thyroid hormones. In contrast, thyroid hormones augment feather loss in birds, which is stimulated by gonadal steroids. These effects of thyroid hormones are similar to their effects on hair replacement in mammals (Norris, 1999).

Thyroid hormones also work synergistically with GH to provide maximal growth rates in fishes, adult amphibians, birds, and possibly reptiles, although the latter have been less studied (Norris, 1997). Finally, thyroid hormones are important stimulators of sexual maturation and are essential for seasonal reproductive events in a wide variety of animals (Norris, 1999). The roles of thyroid hormones in controlling metabolic rates, body temperature, and thermogenesis evolved independently in accordance with homeothermy in both mammals and birds, and these hormones do not play similar roles in fishes, amphibians, and reptiles (Oppenheimer et al., 1995).

3.6 The Pineal Gland: A Photoperiodic Transducer

In mammals, the pineal gland is located above the thalamus of the brain between the cerebral cortices. Through its nocturnal secretion of the biogenic amine melatonin, the pineal has effects on the regulation of many internal physiological rhythms and may provide an important clue for translating photoperiodic stimuli into action. Furthermore, melatonin can alter coat pigmentation and hair growth; can inhibit hypothalamic regulation of the HPA, HPT, and HPG axes; and has been shown to enhance the immune response system (Norris, 1999). Photic input in birds and mammals is accomplished primarily via the optic visual system. However, the pineal of most fishes, amphibians, and reptiles also plays an important role as a direct photoreceptor, and through secretion of melatonin, it may be an important modulator of the HPA, HPT, and HPG axes as well. Any environmental factor that alters pineal function may have profound effects on the well-being of vertebrates.

3.7 Interactions of the HPG Axis with Other Endocrine Systems

The various endocrine axes of the body do not function as isolated islands, as this would clearly compromise the ability of an organism to react and adapt to changing circumstances, for example, season, food supply, and presence of predators. Various components of

other endocrine axes are able to exert important modulatory effects on the HPG axis to alter the timing or efficiency of reproduction. The complexity of such interactions is illustrated in Figure 3.4, which highlights some of the overlapping cross talk. To give further insight into the function and complexity of these interactions, four additional points (with examples) are emphasized.

3.8 Growth in Understanding of Endocrine Systems

New pathways of communication and functional overlap between the various endocrine systems are still being discovered. One example is the relatively recent discovery of leptin following studies of the genetically obese (*ob/ob*) mouse (Rosenbaum and Leibel, 1998). This hormone is produced by adipocytes and exerts important effects on feelings of satiety and hunger and on the appropriate behaviors such as feeding. As fat cell energy reserves are an important component of the insulin–glucose endocrine system, it is known that insulin can exert both direct and indirect (i.e., by altering fat deposition) effects on leptin levels (Figure 3.4). There are also important interactions between leptin and the reproductive system (Friedman and Halaas, 1998; Rosenbaum and Leibel, 1998). An animal reproduces only when the mother has appropriate energy reserves and food supply is good. Leptin provides the necessary signal that unites these various components. When food supply and maternal energy (= fat) reserves are low, elevated leptin can suppress function of the reproductive system. Such pathways play an important role in the timing/initiation of puberty, in regulation of seasonal reproduction, and in certain diseases, for example, the cessation of normal menstrual cycles in women with eating disorders such as anorexia. Although the existence and implications for leptin in nonmammalian vertebrates remain to be explored, the importance of nutritional state to reproduction is known to be as critical as the relationship described in mammals and may exist in all vertebrates.

Another recently recognized complexity of the endocrine system involves the capability of steroids to exert rapid, nongenomic actions by binding to receptors on the cell surface of target cells and activating signal transduction pathways leading to biological responses, in addition to effects mediated by the classic mechanisms of binding to nuclear steroid receptors leading to changes in gene expression (Watson and Gametch, 1999). Specific membrane receptors and rapid nongenomic actions for estrogens and androgens have recently been identified throughout the reproductive system in vertebrates, including the hypothalamus, pituitary, gonads, gametes, steroidogenic cells, and primary and secondary reproductive structures such as the breast (Revelli et al., 1998). Nongenomic steroid actions have been shown to have important functions in several reproductive processes, such as the activation of sperm in mammals (Luconi et al., 2001), electrolyte and fluid transport in the efferent duct of the testes (Leung et al., 2001), and oocyte maturation in fish and amphibians by progestins (Thomas et al., 1998), as well as nonreproductive processes such as chondrocyte proliferation, differentiation, and matrix formation, but their physiological role in most tissues remains unclear. This area is likely to see increased attention in the near future and further complicate the detection and characterization of the full range of effects elicited by EDCs.

3.9 Developmental/Programming Effects of Endocrine Systems

Cross talk between the endocrine systems may have different consequences at different stages of life. Of particular importance are the radical effects that may result from changes to an endocrine axis during the phase when it is "being set up," that is, when thresholds for stimulatory and feedback loops are being programmed. Two such effects can result in changes to the reproductive endocrine axis. The first of these is relatively straightforward and involves the thyroid axis. The circulating level of thyroid hormones (T_3/T_4) can affect terminal differentiation of various tissues (e.g., neurons, muscle cells), and recent studies show that this extends to Sertoli cells in the male. Conversion of the Sertoli cell from an immature, proliferative cell to a mature, nonproliferating cell ready to support spermatogenesis is triggered by thyroid hormone levels in the prepubertal period. Subnormal levels of T_3/T_4 (hypothyroidism) result in prolongation of the Sertoli cell proliferative phase, whereas conversely, supranormal levels of T_3/T_4 (hyperthyroidism) attenuate the Sertoli cell proliferative phase (Sharpe, 1994; Jannini et al., 1995). The net result of such changes is to alter (up or down, respectively) final Sertoli cell number and thereby to alter final testis size and the number of sperm produced per day, because each Sertoli cell can support only a finite number of germ cells. Hypo- and hyperthyroidism have many other consequences in terms of altered body growth and brain development (Figure 3.4), emphasizing again the pleiotropic effects that result from altered function of any individual endocrine axis.

In addition to cross talk, thyroid hormones have important direct effects on differentiation of the brain as well as on its level of activity in adult animals (Akaike et al., 1991; Porterfield and Hendry, 1998). In humans, thyroid deficiencies during gestation or immediately postnatally can produce irreversible mental retardation.

Another, more subtle example of the dramatic consequences of "programming" cross talk between endocrine axes is that of IUGR, which has already been referred to in brief. Small-for-gestational-age offspring are "insulin resistant" as a result of their growth restriction *in utero* and have dramatically lower fat reserves and leptin levels at birth. Such offspring usually exhibit catch-up growth (Jaquet et al., 1999), presumably because of their adapted endocrine changes but remain permanently hyperinsulinemic and insulin resistant. In humans, such individuals are at increased risk of developing diabetes and becoming obese (Philips et al., 1998). Such individuals are also hypertensive and are thus at increased risk of cardiovascular disease, strokes, and kidney disease unrelated to any obesity. The reproductive system is also affected in an adverse way. In the human, IUGR male offspring are at increased risk of cryptorchidism and hypospadias at birth and of developing testicular germ cell cancer and having low sperm counts in adulthood (Sharpe, 1999). Exactly how these changes are induced is unclear but may involve alterations in sex steroid levels. One possible pathway is illustrated in Figure 3.5. It is now well established that hyperinsulinemia results in considerably reduced secretion of SHBG by the liver and a consequent rise in the circulating levels of free (biologically active) sex steroids (Nestler, 1993), and because androgens bind more strongly to SHBG than do estrogens, the androgen/estrogen ratio will be altered. This is dramatically evident in one common disorder of women, polycystic ovarian disease. Such individuals are hyperinsulinemic and have supranormal blood androgen levels that lead to hirsutism as well as polycystic ovaries and anovulatory infertility (Dunaif, 1997). A common risk factor for developing this condition is IUGR.

3.10 Nonreproductive Effects of Sex Steroids

Changes in SHBG resulting from hyperinsulinemia lead to altered androgen and estrogen bioactivity, which, predictably, can alter function of the reproductive system. However, the sex steroids (in

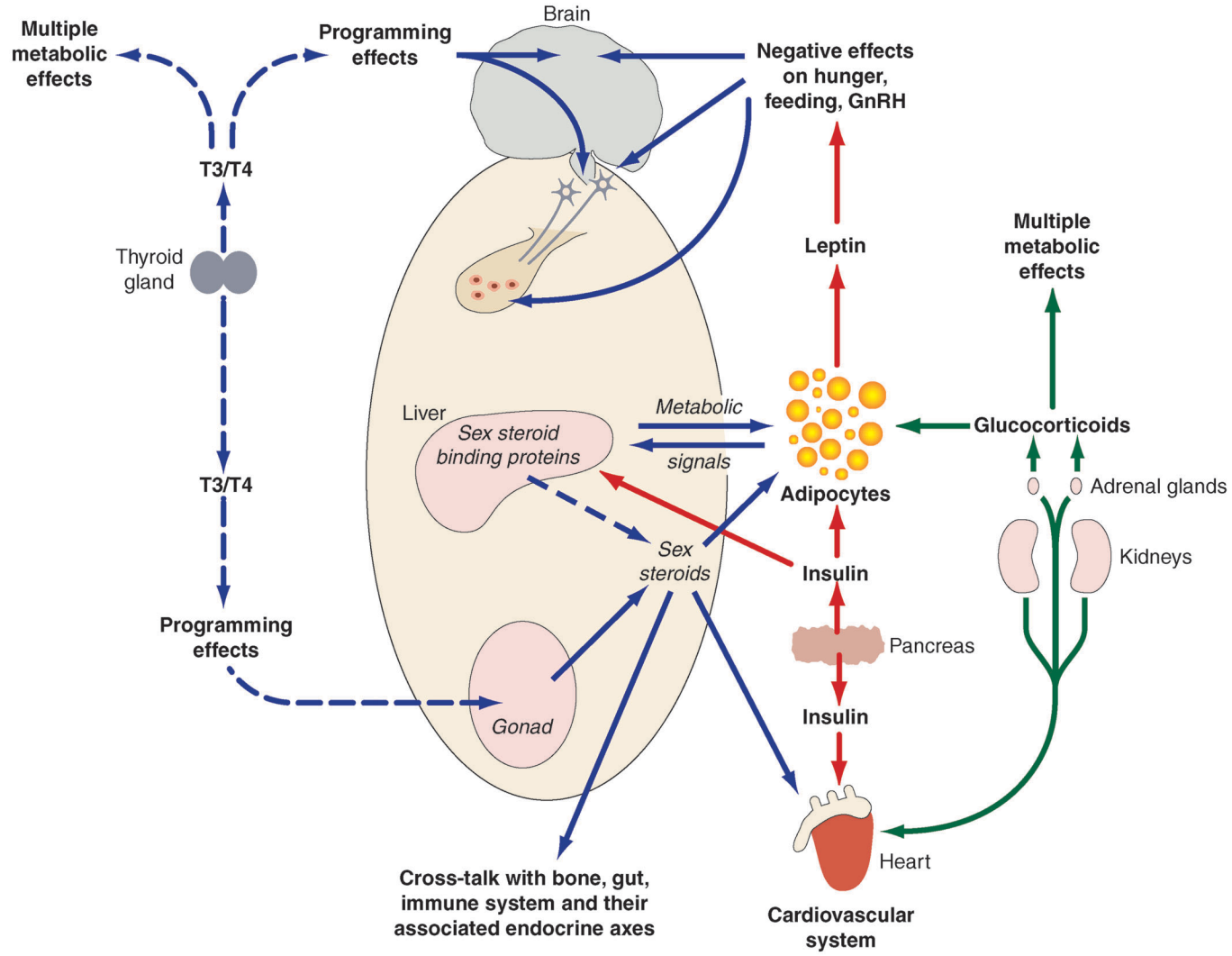


Figure 3.4 - Schematic diagram to illustrate some of the cross talk (integration), which occurs between the mammalian HPG axis (center, shaded) and some of the other endocrine axes of the body. Note that only selective examples are shown and that, in reality, each endocrine axis interacts at multiple levels with other endocrine axes in order to integrate all bodily functions. An important consequence of this complex cross talk is that changes induced in one endocrine axis are likely to lead to changes in other endocrine axes and that such effects can be difficult to predict because of our imperfect understanding of these interactions.

particular, estrogens) also exert multiple other effects throughout the body. Estrogens (and, to a lesser extent, androgens) play a key role in bone formation/resorption in males and females, and estrogen action is essential for epiphyseal closure (Sharpe, 1998). Disorders of sex steroid production or action can lead to osteoporosis or to premature/delayed epiphyseal closure, with consequent effects on final height/body length. Additionally, the sex steroids exert pervasive effects on the cardiovascular system and, in the human, are clearly implicated in gender- and age-dependent changes in risk of developing cardiovascular disease (Sharpe 1998). Multiple effects of the sex steroids on the brain (Gorski, 1996; Meewen and Alves, 1999), digestive system (Sharpe, 1998), immune system (Olsen and Kovacs, 1996), and adipose tissue (Simpson et al., 1997) also occur and, in so doing, will interact with or modulate other endocrine axes that target these tissues (Figure 3.4). Direct or indirect effects on the HPG axis that result in changes in the absolute or relative levels of androgens and estrogens can have pervasive consequences. These effects can be acute/transient (e.g., pituitary feedback effects) or chronic (e.g., bone and cardiovascular effects) in the adult, whereas in the fetus/neonate, any effects that occur may be permanent (e.g., sexual differentiation).

3.11 Endocrine Cross Talk and Endocrine Disruptors

It is emphasized that the prediction of the reproductive consequences of a given chemical from its known sex steroid hormone activity or inactivity is far from straightforward. For example, a dietary change that affects insulin levels (e.g., ingestion of a diet rich in refined sugars) has the potential to alter sex steroid hormone bioactivity via altered production of SHBG (Figure 3.5), but it would not be claimed that refined sugars have sex steroid hormone activity. Even when an environmental chemical is shown to have (weak) steroid hormone activity, it can possess other relevant activities. Thus, the thyroidal activity of PCBs may be as important or even more important than the weak estrogenic/antiestrogenic effects of these compounds when considering their potential impact on the reproductive system. Other environmental chemicals may have antiandrogenic as well as estrogenic effects (e.g., DDT isomers, certain phthalates), which may confuse interpretation of potency *in vivo*. For example, administration of antiandrogens is likely to elevate endogenous estrogen levels. This occurs because antiandrogens block negative feedback loops, which leads to

compensatory elevation of LH levels (Figures 3.1 and 3.2) and thus to supranormal elevation of testosterone levels, with a consequent increase in availability of substrate for aromatization (Figure 3.3). If androgens positively regulate aromatase expression, as various studies suggest (Simpson et al., 1997), further elevation of estrogen levels will occur. Thus, the overall "estrogenic" activity of PCBs, DDT, or phthalates *in vivo* could not be predicted from measurement of their activity in any *in vitro* estrogen screening system. Some estrogens are agonists in one tissue and antagonists in another (e.g., tamoxifen, raloxifene), and emerging understanding suggests that the same will hold true for androgens. Although the basis for these differences is not understood completely, it is clear that tissue-specific expression of co-activator proteins (or adapters) is involved and, in the case of estrogens, whether ER-α and/or ER-β is expressed. As some co-activators may be shared between various members of the steroid receptor superfamily, action of one member might alter availability of co-activators to interact with androgen- or estrogen-receptor complexes. Alternatively, nonreproductive hormones might regulate the expression of these co-activators. Although these possibilities are speculative, the insights afforded by tamoxifen, raloxifene, and other emerging SERMs (Cosman and Lindsay, 1999) emphasize the unpredictable pathways by which sex steroid hormone action can be altered.

Based on the considerations detailed above, it is clear that chemicals should be tested for their "reproductive" activity (i.e., the ability to alter the development or the function of the reproductive system) rather than just for their sex steroid activity in *in vitro* tests systems if the objective is to establish whether or not the compound in question is a reproductive endocrine disruptor.

3.12 Modes of Action and Phenotypic Outcomes of EDC-Related Developmental and Reproductive Toxicities

3.12.1 Scope of Survey

This section presents several selected examples of the mechanisms of action of several well-characterized EDCs that have been shown to produce adverse effects *in vivo* on reproductive function and development. As expected from the normal functioning of endocrine systems, the cellular and molecular mechanisms of endocrine disruption are not limited to receptor binding and include, for example, inhibition of hormone synthesis, metabolism, and transport. Figure 3.6 displays some of the mechanisms of steroid hormone action, as an example, and provides key steps in the process where EDCs have been shown to alter endocrine function. Other sites in the signal transduction process are also likely to be susceptible to disruption by anthropogenic chemicals. This section highlights the cellular and molecular mechanisms of action and toxicological impact on the developing reproductive system using selected examples of steroid receptor agonists and antagonists, steroid synthesis modifiers, and AhR agonists. When available, data from nonmammalian species are also presented. EDCs typically alter reproductive development by more than one mechanism such that several target organs are impacted, although not necessarily at the same dose or same precise stage of life. It is critical to recognize the limitations of *in vitro* tests, as the multiple endocrine and nonendocrine effects of a toxicant can only be interpreted in a meaningful and comprehensive fashion *in vivo*.

Considerable homology exists in the endocrinology of vertebrates; hence, toxicants that alter endocrine function in one species are likely to produce adverse effects in another. However, there are significant differences between some species in endocrine function that warrant consideration for further interspecies extrapolations. Although the hormones, hormone synthesis, and their receptors are highly conserved, the role of specific hormones in reproductive function and development can vary greatly. Additionally, significant differences in metabolism of EDCs can result in marked species differences in responses to these chemicals.

3.12.2 AR-Mediated (Anti)Androgens

3.12.2.1 Vinclozolin. It is generally assumed that mammals possess a single AR, as evidenced by the complete phenotypic sex reversal displayed in humans with androgen insufficiency syndrome as a consequence of a single base substitution in the AR (Quigley et al., 1995). Vinclozolin is a dicarboximide fungicide with AR antagonism. Of the EDCs, the cellular and molecular mechanisms of action of the antiandrogenic fungicide vinclozolin are one of the most thoroughly characterized. Vinclozolin metabolites M1 and M2 competitively inhibit the binding of androgens to the mammalian

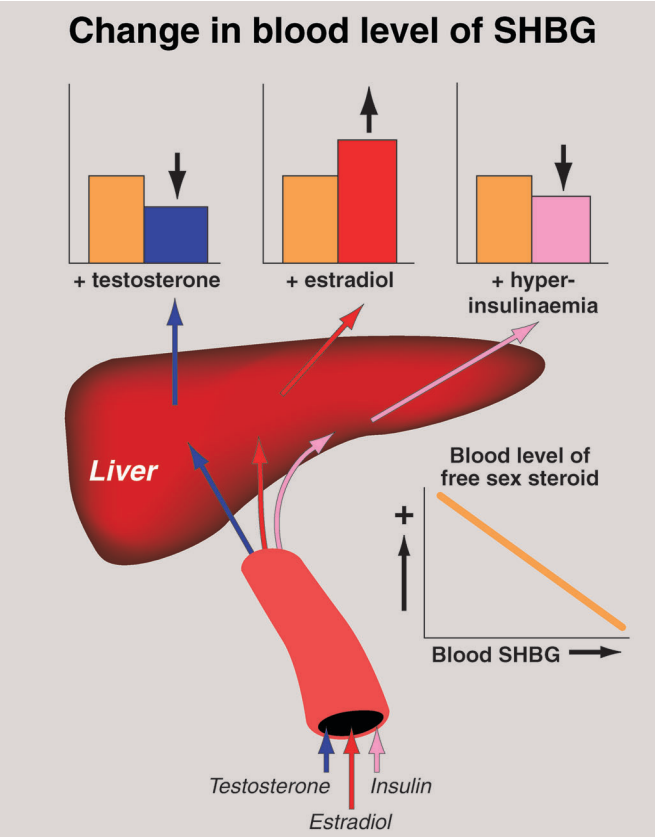


Figure 3.5 Diagrammatic illustration of one potentially important example of cross talk between endocrine axes in the human with particular relevance to endocrine disruption. In humans and some other mammals, the sex steroids testosterone and E_2 circulate in the bloodstream bound to SHBG and are thus not freely available to target cells. Variation in the levels of SHBG (produced by the liver) will alter the biological activity of testosterone and E_2 without altering their production. Perhaps not surprising, SHBG production is itself regulated by testosterone (negatively) and E_2 (positively). However, via cross talk, elevated levels of insulin also lead to suppression of SHBG production and, as a consequence, will increase the level of biologically active (= not bound to SHBG) testosterone and E_2. Moreover, because testosterone binds more strongly to SHBG than does E_2, a decrease in SHBG levels leads to a preferentially greater rise in bioactive testosterone and thus alters the androgen/estrogen balance. Factors that lead to elevation of blood insulin levels, such as a sugar-rich diet, can therefore potentially alter levels and actions of the sex steroids on target tissues.

AR. M1 and M2 also inhibit DHT-induced transcriptional activity in cells transfected with the human AR. Kelce et al. (1997) demonstrated that vinclozolin treatment altered gene expression *in vivo* in an antiandrogenic manner. In contrast to the binding to the AR, neither vinclozolin nor its antiandrogenic metabolites display affinity for the ER, although they do have weak affinity for the progesterone receptor. Vinclozolin, M1, and M2 do not inhibit 5α-reductase activity *in vitro*, the enzyme required for the conversion of testosterone to the more active androgen DHT (Kelce et al., 1994). A comparison of the *in vivo* and *in vitro* dosimetry data with the biological effects of vinclozolin reveals that when M1 and M2 concentrations in maternal serum approach their respective K_i values for AR binding, male offspring are malformed (Kelce et al., 1994; Monosson et al., 1999).

The ability of M1 and M2 to inhibit AR-dependent gene expression has been demonstrated both *in vitro* and *in vivo*. In addition, vinclozolin inhibits growth of androgen-dependent tissues in the castrate-immature-testosterone-treated male rat, a further demonstration of its antiandrogenic action *in vivo*. The drug flutamide is metabolically activated to hydroxyflutamide, which is similar in structure to the vinclozolin metabolite M2 and exhibits endocrine activity that is nearly identical to vinclozolin or M2, respectively (Imperato-McGinley et al., 1992; Gray et al., 1994; Kelce et al., 1995).

In addition to their antiandrogenic effects on the reproductive tract, vinclozolin and flutamide alter reproductive function at the level of the hypothalamic-pituitary axis. Oral treatment with vinclozolin (30–100 mg/kg/day; Monosson et al., 1999) or flutamide causes elevations of serum LH and testosterone levels and Leydig cell hyperplasia. In contrast to vinclozolin and flutamide, treatment with *p,p´*-DDE (Kelce et al., 1995) or MXC (Gray et al., 1989, 1999c), which are also antiandrogenic, fails to induce any significant change in serum LH or testosterone levels. A wide variety of antiandrogenic teratogenic effects in male offspring are noted following late gestational exposure to dose levels of vinclozolin in the range of 3–200 mg/kg/day (Gray et al., 1994, 1999b). These include a femalelike AGD, retained nipples, cleft phallus with hypospadias, suprainguinal ectopic testes, vaginal pouch, epididymal granulomas and small or absent accessory sex glands and delays in preputial separation. In a study of the low-dose effects of vinclozolin, pregnant rats were exposed to between 3 and 100 mg/kg/day from gestation day 14 to postnatal day 3. Even the lowest dose group (3.125 mg/kg/day) produced significant effects on AGD and retention of nipples in male offspring. Malformations of the male reproductive tract were observed at 50 and 100 mg/kg/day. Even though all of these end points (reduced AGD, retained nipples, effects on accessory sex gland weight, hypospadias, epididymal agenesis) are believed to be elicited by interference at the level of the AR, they display a wide variety of effective dose levels producing statistically significant changes. Some of these changes do not exhibit an obvious threshold in the range of the experimental dose levels. Multigenerational studies are essential to detect subtle antiandrogenic effects on male reproduction, and a failure to utilize the new testing guidelines (which incorporate new antiandrogenic measures) could yield NOAELs at least an order of magnitude too high.

Dermal exposure of adolescent rabbits to 100 mg/kg of vinclozolin for 2 months resulted in reduced accessory gland weights, but sperm counts were significantly elevated. The authors suggested that the antiandrogenic effects may have blocked the negative feedback of testosterone on the hypothalamus or pituitary allowing for increased gonadotropin release (Moorman et al., 2000).

3.12.2.2 Other AR antagonists. Several other toxic substances have been shown to display AR-antagonist activity, including the DDT metabolite DDE (Kelce et al., 1995; Gray et al., 1999a; You et al., 1998, 1999a, 1999b), the MXC metabolite HPTE (Gaido et al., 1999; Maness et al., 1998), the organophosphate fenitrothion (Tamura et al., 2001), and the dicarboximide fungicide procymidone (Ostby et al., 1999). Linuron is a urea-based herbicide that displays weak affinity for the AR, but the effects induced in male offspring indicate that it may alter mammalian sex differentiation via more than one mechanism of action (Gray et al., 1999a; Lambright et al., 2000; McIntyre et al., 2000). In this regard, the phytoantiandrogenic drug permixon, used clinically for prostate problems, appears to bind AR and inhibit steroid hormone synthesis as well (Carilla et al., 1984; Bayne et al., 1999; Plosker and Brogden, 1996). Tris(4-chlorophenyl)methanol is a global contaminant of unknown origin that is structurally related to DDT, has binding affinity for the AR comparable to *p,p´*-DDE. However, it has not demonstrated antiandrogenic effects *in vivo* when sexually mature rats were exposed for 28 days at doses up to 100 ppm in the diet (Foster et al., 1999).

3.12.2.3 AR-mediated effects among other vertebrates. Sexual dimorphisms in fish have been demonstrated to be affected by both androgens and estrogens (Ankley et al., 1998). For example, Smith (1974) demonstrated that the formation of breeding tubercles and a mucus-secreting dorsal pad in the fathead minnow is inducible by 17α-methyltestosterone. When aromatizable (testosterone and 17α-methyltestosterone) and nonaromatizable (11-ketotestosterone and 17α-methyl-DHT) were administered to newly hatched genotypic female chinook salmon for 2 hours, dose-dependent sex reversal was observed, with the synthetic and nonaromatizable forms more potent than the natural or aromatizable forms, thus indicating a role for aromatase early in development (Piferrer et al., 1993). Predominantly male populations of tilapia can be produced on a commercial scale by feeding androgens to fry. Administration of 25 mg of trenboline acetate for 28 days to sexually undifferentiated *Oncorhynchus aureus* resulted in 98% phenotypic males (vs. 55.7% males in the control group; higher exposures yielded lower percentages of males, presumably due to the less potent antiestrogenic activity (Galvez et al., 1996). In similarly treated channel catfish, Davis et al. (2000) found evidence that adults were lighter and shorter and had smaller gonads and GSI and lower plasma testosterone as adults than did control males. When the reproductively mature fathead minnows were exposed to methyltestosterone for 21 days, a decrease in plasma concentrations of sex steroids and adversely affected gonadal status (as evidenced by relative weight and histopathology) was observed in both sexes (Ankley et al., 2001). The androgenic nature of methyltestosterone was clearly evidenced by masculinization of exposed females. Vitellogenin induction was observed in both sexes, probably as result of aromatization of the administered androgen. Although mammals are believed to possess a single AR (Quigley et al., 1995), some piscivorous species have two ARs, termed AR1 and AR2 (Sperry and Thomas, 1999a, 1999b). AR1 in the brain displays binding affinities for ligands quite distinct from AR2, which has similar ligand affinities to mammalian AR. AR2 has been shown to bind *p,p´*-DDE and vinclozolin metabolites M1 and M2 (Sperry and Thomas, 1999a), demonstrating the homology of AR function *in vitro* among diverse species of vertebrates. *In vivo*, vinclozolin treatment induces intersex in the medaka (*Oryzias latipes*), which displays a mammalian-type sex differentiation (Koger at al 1999). In contrast, Makynen et al. (2000) did not obtain sex reversal in the fathead minnow with vinclozolin

treatment. This may be related to several factors, including a lack of metabolic activation of vinclozolin (Makynen et al., 2000) and the undefined role for androgens in the sex differentiation process. However, in contrast to the results cited above, M1 and M2 did not bind AR in this species (Makynen et al., 2000).

Takeo and Yamashita (1999) described two distinct rainbow trout cDNA clones, which were designated rtAR-α and rtAR-β, that contain the entire AR coding region. Comparison of the predicted amino acid sequence of rtAR-α to that of rtAR-β revealed 85% identity. Despite this high homology, rtAR-α activated transcription of an androgen-responsive reporter gene in cotransfection assays, but rtAR-β did not, suggesting that rainbow trout contains two distinct isoforms of ARs whose functions differ.

Ikeuchi et al. (1999) identified 11-ketotestosterone (a potent androgen in teleosts) as the spermatogenesis-inducing hormone of the Japanese eel and cloned its receptor (eAR1) cDNA from eel testis, and also reported that they cloned a second type of AR (eAR2) from the eel testis, encoding 797 amino acid residues. The amino acid sequence of eAR2 shows high homology with other ARs, including eAR1, in the DNA-binding (98–88%) and ligand-binding (59–85%) domains, whereas the other domains show low homology. In transient transfection assays of mammalian cells, the eAR2 displayed androgen-dependent activation of transcription from the androgen-responsive murine mammary tumor virus promoter. Tissue distribution of its mRNA differed from that of eAR1. The degree to which the differences in amino acid sequences in the ligand binding domain between ARs of lower vertebrates and mammalian AR affect binding of natural and synthetic ligands remains to be determined. Although it is clear that differences in overall sequence homology of this region exist, it appears that the amino acids in the binding pocket of the human AR that result in loss of function when mutated (Quigley et al., 1995) are more highly conserved in the fish ARs than are other amino acids in this domain.

3.12.2.4 Other sites of action of antiandrogens: liver and adrenal. Although vinclozolin and its metabolites do not bind the glucocorticoid receptor (Kelce et al., 1994), vinclozolin treatment has been shown to alter the pituitary-adrenal axis in several mammalian species, including rats (Monosson et al., 1999) and dogs. Indeed, the former no adverse effect level (NOAEL) was established on the effects of vinclozolin on the canine adrenal gland. The effects of vinclozolin (Monosson et al., 1999) and flutamide (Wada et al., 1999; Migliari et al., 1999) on liver function are also noteworthy. Although the mechanism of action for the hepatic effects of these antiandrogens has not been elucidated, androgens and antiandrogens acting via the AR are known to alter several aspects of liver growth and metabolism. In particular, cyproterone acetate, a drug that has antiandrogenic, progestational, and antiglucocorticoid activity, also acts as a liver mitogen (Kasper et al., 1999). The mechanisms of action of vinclozolin and flutamide on the liver warrant study, as these chemicals induce adverse effects with long-term treatment (flutamide-induced morbidity due to liver failure and vinclozolin-induced liver tumors in male rats).

3.12.3 ER-Mediated Estrogens

3.12.3.1 Overview. The ability of pesticides to act as estrogen agonists, inducing a uterotropic response, has been known for over 30 years (Bittman et al., 1968), and the estrogenicity of anthropogenic chemicals, for example, bisphenol A and DES, were first described in 1938 (Dodds and Lawson, 1938). Many estrogens have been identified using *in vitro* assays (e.g., ER binding, breast

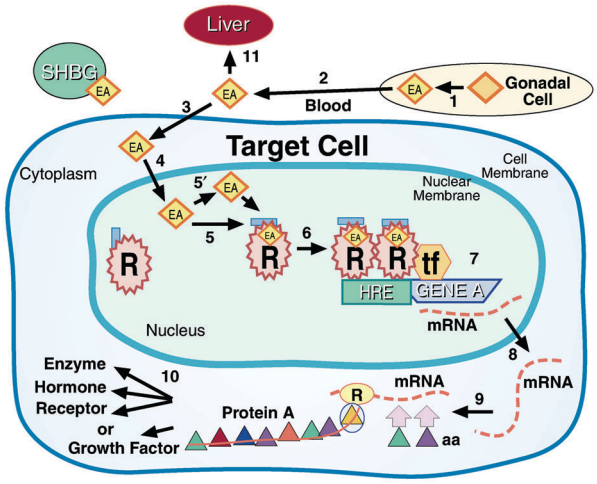


Figure 3.6 - Schematic diagram depicting several key steps in steroid hormone action that may be sensitive to disruption by environmental chemicals:

(1) Steroid hormones (EA) such as E_2, testosterone, and progesterone are synthesized in the gonadal cells. Inhibitors of CYP450 enzymes, including drugs and pesticides, act here.

(2) Hormones are secreted in the blood from the gonad and are available to the cell through diffusion or may be transported bound to SHBG. The degree of free and bound hormone depends upon several factors, but bound hormone disassociates from SHBG at a rate that depends on the binding affinity of the steroid for the SHBG protein. Toxicants could alter SHBG levels, and it is reported that some hormone mimicks do not bind SHBG as well as the natural ligands do, making them more available both to the target cell and for liver metabolism.

(3) The steroid hormone diffuses into the cell.

(4) The hormone diffuses into the perinuclear region, where unoccupied receptors (R) are located.

(5) The hormone, or hormone mimic, binds the receptor. Many xenobiotics have been shown to bind ER or AR. In some cases, the chemical secreted into the blood is a prohormone, that is metabolized in the cell (5′ to the active hormone. For example, in some tissues, testosterone is metabolized by aromatase to E_2, whereas in others the enzyme 5α-reductase converts it to DHT. In some tissues, such as muscle, testosterone itself is the active hormone. Some EDCs inhibit the activation of the prohormone in the target tissue.

(6) The receptor (R), now bound to a natural or synthetic ligand, undergoes a conformational change, exposing key protein binding sites, and forms homodimers.

(7) The homodimers, accumulate transcriptional factors (tf), forming a transcriptional complex, which binds to specific sequences on the DNA of hormone-dependent genes, known as hormone response elements (HRE). The transcriptional complex then initiates mRNA synthesis (mRNA). Some antihormones interfere with DNA binding.

(8) mRNA is transported out of the cell into the cytoplasm.

(9) In association with amino acids (aa) bound to specific tRNAs (the thick arrows) and ribosomes, proteins (the circles on a "string") are synthesized from the mRNA template.

(10) The protein, a marker of endocrine action, could be an enzyme, a protein hormone or growth factor, or a structural component of the cell. An example of a hormone-dependent marker is vitellogenin, an estrogen-sensitive protein produced by oviparous vertebrates.

(11) In some cases, toxicants disrupt endocrine function by altering liver function, either increasing or decreasing metabolism of the hormone, such that serum levels are altered. For example, some PCB stimulate metabolism of T_4, dramatically reducing serum T_4 levels. Several pesticides have been shown to stimulate the liver and reduce serum steroid hormone levels.

cancer cell proliferation, and transcriptional activation), and several also display estrogenic activity *in vivo,* including MXC, chlordecone, octylphenol, nonylphenol, bisphenol A and B, phytoestrogens (genistein), ethynyl estradiol, and fungal mycotoxins (zearalenone). Other chemicals that have shown evidence *in vitro* of estrogenic activity have not shown similar evidence in *in vivo* systems, and caution is warranted in interpreting *in vitro* results without *in vivo* confirmation. *In vitro*, some estrogens, such as bisphenol A and E_2, have been reported to interact in an unanticipated manner, with bisphenol A antagonizing some of the effects of E_2 (Gould et al., 1998). Phytoestrogens present in a variety of plants such as soy (isoflavanoids) and berries, fruits, grains, vegetables, and nuts (lignans) represent another source of exposure to estrogenic chemicals (reviewed in Whitten and Patisaul, 2001). Binding studies show that isoflavanoid phytoestrogens are high-affinity ligands for ERs, especially ER-β, but have lower potencies *in vitro* cell-based assays. *In vivo* data indicate that phytoestrogens have a wide range of biologic effects at doses and plasma concentrations seen with normal human diets. *In vivo* data effects have been reported for bone, ovary, pituitary, vasculature, prostate, and serum lipids. Effective doses in humans (0.4–10 mg/kg/day) are generally lower than those causing effects in rodents (10–100 mg/kg/day), although careful pharmacokinetic comparisons of circulating dose are not available to truly compare the species sensitivity.

3.12.3.2 MXC: an estrogenic and antiandrogenic pesticide. The estrogenic pesticide MXC is still in commercial use. This DDT derivative usually does not bioaccumulate because it can be metabolized by some species more readily than the metabolites of DDT. MXC provides an example of the multiplicity of EDC action because it is an ER-α agonist, an ER-β antagonist (Maness et al., 1998; Gaido et al., 1999), and an AR antagonist. *In vivo*, MXC displays estrogenic ER-α–mediated activity in many tissues, including the uterus, vagina, brain (behavior), and bone, but not in the hypothalamic-pituitary axis. MXC treatment failed to induce hyperprolactinemia, inhibit LH, or induce pituitary tumors in the rat after long-term high-dose treatment (Gray et al., 1988, 1989, 1999c). In the adult and pubertal male rat, MXC antagonizes the effects of androgens, which may indicate that MXC metabolites are inhibiting testosterone and DHT-induced gene expression and tissue growth and differentiation, as some of these tissues lack ER. Many natural and anthropogenic estrogens display affinity for AR, acting as AR antagonists and agonists in *in vitro* assays (Danzo, 1997; Baker et al., 1999), albeit often at high concentrations.

MXC itself is weakly active or inactive *in vitro* in ER binding and transcriptional activation assays. Purer forms of MXC (>99%) are inactive compared with less pure MXC (>95% pure) (Bulger et al., 1978a, 1978b). MXC is metabolically activated to several monohydroxy and dihydroxy metabolites that display estrogenic activity. One of these, HPTE also is a relatively potent AR and ER-β antagonist (Maness et al., 1998; Gaido et al., 1999) as well as an ER-α agonist.

Treatment of adult male rats with MXC alters fertility at very high doses by the inhibition of spermatogenesis (Gray et al., 1999c); lower dose levels (~25–200 mg/kg/day) reduce epididymal sperm reserves and seminal vesicle weight without affecting sperm production, testicular morphology, or serum testosterone levels. If treatment is administered at weaning, MXC will induce a number of effects in male offspring, including delays in puberty and reduced accessory sex gland weights. In the adult female, MXC induces effects more typical of an estrogen, with induction of lordosis. There were no effects on circulating LH or testosterone in the presence of delayed puberty, but there was an elevation in serum PRL. The lack of an effect on LH may be suggestive that, rather than working through the pituitary, the agent may have a direct effect on the reproductive tract. In longer term exposure, dosing of MXC for 10 months (200–400 mg/kg/day) to male rats delayed puberty by up to 10 weeks, reduced fertility, and altered reproductive behavior but did not mimic the chronic sustained effects of E_2 given via Silastic implants. In a study in which MXC was given to female rats by gavage at 0, 5, 50, or 150 mg/kg/day for the week before and the week after birth, with the pups then directly dosed with MXC from postnatal day 7 (Chapin et al., 1997a), the high dose of MXC reduced litter size by approximately 17%. AGD was unchanged, although male prepuce separation was delayed at the middle and high doses by 8 and 34 days, respectively. High-dose males impregnated fewer untreated females; epididymal sperm count and testis weight were reduced at the high and top two doses, respectively. Female effects (vaginal opening, estrous cyclicity) were noted at 50 mg/kg/day and above.

3.12.3.3 Mechanisms of action of xenoestrogens in other vertebrates. Several of the xenoestrogens bind one of the fish ERs with affinity similar to that displayed for mammalian ER (Loomis and Thomas, 1999). Octylphenol, nonylphenol, bisphenol A, *o,p*-DDT, ethynyl estradiol, and MXC display estrogenic activity in lower vertebrates (i.e., fish and frogs). In avian and mammalian species, *o,p´*-DDT, but not *p,p*-DDT (Bitmann et al., 1968), induced growth of the female reproductive tract.

Some of these xenoestrogens induce gonadal intersex (Kloas et al., 1999), vitellogenin synthesis in males (Kloas et al., 1999; Lutz and Kloas, 1999), and hermaphroditism and estrogen-dependent sexual dimorphisms (Noriega and Hayes, 2000). The toxicants that induce estrogen-dependent changes in coloration in the African reed frog (*Hyperolius argus;* Hayes, 1998a; Hayes and Menendez, 1999) are remarkably similar to those that induce a uterotropic response in the female rat, indicating a high degree of homology in ER-α function despite the differences in ER sequence.

In contrast, the estrogenicity of MXC is likely to be as widespread throughout the animal kingdom because it will not be estrogenic in those lower vertebrate species that are unable to metabolically activate it. Hydroxylation of MXC is required for estrogenicity and for excretion; hence, these species that cannot metabolically active MXC have a tendency to bioaccumulate this pesticide to the same degree as DDT. In male catfish pretreated with MXC or BNF alone or in combination, pretreatment with MXC but not BNF significantly reduced rates of MXC biotransformation, and pretreatment with MXC/BNF followed by MXC significantly induced serum vitellogenin, whereas MXC alone did not significantly elevate vitellogenin, thus demonstrating in this species that MXC can elicit estrogenic activity despite diminished capacity to form estrogenic metabolites (Schlenk et al., 1998). In a short-term reproduction test with the fathead minnow (*Pimepehales promelas*) initiated with reproductively mature animals exposed for up to 21 days, MXC decreased plasma concentrations of one or more steroids (testosterone, 11-ketotestosterone, E_2) in both sexes and caused a significant induction of plasma vitellogenin in males (Ankley et al., 2001). A significant decrease in fecundity was also observed at the same concentration that induced vitellogenin (3.56 µg/liter). Continuous exposure of adult male sheepshead minnow (*Cyprinodin variegates*) to *p*-nonylphenol, MXC, or endosulfan for up to 42 days was observed to induce a dose-dependent increase in hepatic vitellogenin mRNA and plasma protein within 5 days of

exposure to all but endosulfan (Hemmer et al., 2001). The fact that MXC, but not its estrogenic metabolite HPTE, alters amphibian germinal vesicle breakdown, which is dependent upon cell-surface hydroxyprogesterone receptor activation, indicates that this effect is not mediated by the ER. As noted in section 3.8, recent studies have shown that a variety of xenobiotic chemicals, in addition the disrupting genomic steroid actions, can also interfere with the nongenomic actions of steroids (Thomas, 1999). The finding that low concentrations (100 nM, equivalent to 30–40 ppb) of the estrogenic compounds kepone and *o,p´*-DDE interfered with progestogen induction of meiotic maturation of Atlantic croaker oocytes *in vitro* provided initial evidence for this novel type of endocrine disruption (Ghosh and Thomas, 1995). Subsequently, disruption of oocyte maturation by estrogenic compounds was confirmed in an amphibian species, *Xenopus*, exposed to MXC (Pickford and Morris, 1999). In addition, kepone was shown to partially block the stimulatory actions of progestogens on sperm motility in Atlantic croaker (Thomas et al., 1998). Estrogenic compounds such as *o,p′*-DDT and nonylphenol have also been shown to exert rapid estrogenic (agonistic) actions on rat smooth muscle cells and on croaker testicular androgen production (Ruehlmann et al., 1998; Loomis and Thomas, 2000). Recently, direct evidence has been obtained that estrogenic compounds can disrupt nongenomic steroid actions by receptor-mediated mechanisms (Thomas, 1999). Competition studies have shown that these compounds bind to the progestogen membrane receptors on fish oocytes and sperm and to the estrogen membrane receptor in fish testes as well as disrupting nongenomic steroid actions in these tissues (Das and Thomas, 1999; Thomas et al., 1998; Loomis and Thomas, 2000).

When a genetically male population of the common carp (*Cyprinus carpio*) was exposed to 4-*tert*-pentylphenol, no effects on sexual differentiation or proliferation of primordial germ cells were evident following 3 days of exposure during the embryo-larval period. However, longer exposures, starting before and including sexual differentiation, induced the formation of an oviduct that was persistent upon returning to clean water. Exposure during these times (days 24–51 posthatch) reduced the number of primordial germ cells (Gimeno et al., 1997). Bisphenol A was observed to increase vitellogenin levels in male rainbow trout (*O. mykiss*) following 14 days of exposure to 500 µg/liter, whereas constant or decreasing levels were present at lower exposure levels. However, the ratio of responding to nonresponding animals indicated that levels as low as 70 µg/liter were effective. Average liver concentration at the 500 µg/liter exposure level was 4.36 µg/g (Lindholst et al., 2000).

A comparative study of 44 PCBs, 9 hydroxylated PCBs, and 8 arochlors binding to the cloned reptilian (*Anolis carolinensis*), recloned rainbow trout (*O. mykiss*), and human ER linked to the glutathione *S*-transferase protein found that only three PCBs (104, 184, and 188) effectively competed with E_2 for binding to the fusion protein from all three species. Data from the reptilian and humans were more similar to each other than to the rainbow trout receptor (Matthews et al., 2000). Five of the mono-*ortho* PCBs (58, 60, 68, 70, and 74) and 9 of 18 di-*ortho*-substituted congeners (18, 44, 49, 99, 101, 112, 128, 138, and 153) weakly interacted, and three others (41, 47, and 115) exhibited moderate binding with the rainbow trout receptor. Similarly, the 13 tri-*ortho* congeners interacted only with the rainbow trout ER. These data suggest that significant differences in relative affinity for ligands may exist across vertebrate steroid receptors.

3.12.4 Inhibitors of Steroid Hormone Synthesis

3.12.4.1 Overview. Several classes of fungicides have been developed to inhibit fungal membrane synthesis and growth by inhibiting specific CYP450 enzymes, especially 14α demethylation of lanosterol, in the sterol pathways. The process of steroidogenesis is sufficiently conserved that these chemicals can also inhibit mammalian steroidogenesis. There are several CYP450 enzymes in the steroid pathway, and the binding affinity for each varies from chemical to chemical. In general, however, at relatively high concentrations, these are nonspecific inhibitors of CYP450 enzymes. Hence, effects are not limited to the reproductive system and include adrenal and liver steroid metabolism in mammals and ecdysteroid synthesis in invertebrates (Schurmeryer and Nieschlag, 1984; Pepper et al., 1990; Williams et al., 2000).

3.12.4.2 Ketoconazole. The antifungal imidazole derivative ketoconazole inhibits various enzymes that belong to the CYP450-dependent mono-oxygenases in rodents and humans such as side chain cleavage of cholesterol and 11β-hydroxylase in the adrenal and 17α-hydroxylase and C_{17-20} lyase in rat and human testes (Schurmeyer and Nieschlag, 1984; Pepper et al., 1990). For example, human testicular mono-oxygenase activities *in vitro* are reduced by 50% by 3.1 µM ketoconazole. Side effects of the use of ketoconazole as a therapeutic antiandrogen include gynecomastia. However, the effects on steroidogenesis are not selective for the testis, with ovary and adrenal effects reported. Effects on adult Leydig cell function have been noted in humans and rodents *in vitro*. When administered to adult rodents, ketoconazole can have dramatic effects on fertility even after a single dose (Bhasi et al., 1986; Heckman et al., 1992; Waller et al., 1990). It is more difficult to observe effects on male reproductive development resulting from a decreased testosterone production, as other effects on ovarian and uterine steroid responses tend to interfere with pregnancy maintenance. Treatment of pregnant dams with ketoconazole is more likely to affect the ability to maintain pregnancy due to effects on ovarian progesterone synthesis resulting in abortion/litter loss that would preclude the observation of marked effects on the pups (Gray et al., 1999a).

3.12.4.3 Aromatase inhibitors. Aromatase CYP450 converts C19 androgens to aromatic C18 estrogens. A number of pharmaceutical agents have been developed that inhibit aromatase, which have been applied as treatment for postmenopausal breast cancer (Brodie et al., 1999). This P450 enzyme is highly conserved in a wide variety of tissues and many species, but the overall homology of the genes (as there is more than one gene) with other CYP450s is only about 30%. Hence, this enzyme is considered to be in a separate gene family within the overall superfamily. A consequence of the lack of sequence homology with other P450 enzymes in the steroid pathway, inhibitors of aromatase may display greater specificity than drugs such as ketaconazole. A yeast-based screening assay has been proposed to test for this activity (Mak et al., 1999). The induction of imposex in mollusks exposed to tributyl tin has been linked to inhibition of aromatase and subsequent estrogen deficiency and enhanced androgen levels. Several fungicides inhibit aromatase activity in mammals, resulting in infertility in both sexes. Fenarimol treatment inhibits male rat mating behavior, presumably by inhibiting the conversion of androgens to estrogens in the brain (Hirsch et al., 1987; Gray et al., 1998), and also inhibits parturition because of the critical role of E_2 near term in the induction of labor. The effects of fenarimol in mammals differ from those seen with ketoconazole, because fenarimol does not inhibit androgen production in male rat or progesterone synthesis during pregnancy.

Fenarimol also has been shown to inhibit ecdysteroid synthesis in invertebrates, whereas in reptiles aromatase inhibitors inhibit female gonadal sex determination (Williams et al., 2000).

Fenarimol caused a dose-related decrease in male fertility in Wistar rats, with the effect particularly evident in the anatomically normal progeny of dams treated with fenarimol throughout life, including gestation and lactation (Hirsch et al., 1987). Based on the observation that the infertility was associated with the absence of vaginal sperm at the time of mating, the effect appeared to be the result of an absence of male sexual behavior. Gray and Ostby (1998) subsequently described a dose-related decrease in male mating behavior of rats when fenarimol was administered daily from weaning through adulthood. These results suggest that fenarimol is acting centrally to decrease male sexual behavior by inhibiting the conversion of testosterone to E_2 in the brain. Consistent with a central effect, Hirsch et al. (1987) noted that fenarimol concentrations in the brain of neonates whose mothers were treated were three- to fourfold higher, and half-life was four times longer, than in other brain regions.

Exposure of genetic stocks of all female chinook salmon (*O. tshawytscha*) to fadrozole [5-(5,6,7,8-tetrahyroimidazo[1,5-α]-pyridin-5yl) benzonitrile monohyrochloride, or CGS 16949A], a nonsteroidal aromatase inhibitor, for a 2-hour period when the gonads were totipotent caused genetic females to develop as males. Resulting males had testes that were indistinguishable in both size and function from genetic males, and were fertile (Piferrer et al., 1994). When fadrozole was given to adult female coho salmon (*O. kisutch*), a lowering in plasma E_2 was observed, associated with an increase in plasma 17α,20β-P. Ten days after injection, 67% of the fish exposed to 10 mg of fadrozole/kg body weight had ovulated, in contrast to 0% in the control group (Afonso et al., 1999), indicating an advancement in oocyte maturation and subsequent ovulation. Administration of fadrozole to male coho salmon during sexual maturation inhibited secretion of E_2 by the brain and increased plasma 17α,20β-P, and treated males began spermiation earlier than control males. In addition, fadrozole-treated fish had higher levels of testosterone and 11-ketotestosterone than did controls within 4 days of injection (Afonso et al., 2000).

3.12.4.4 5α-Reductase inhibitors. 5α-Reductase is the enzyme responsible for the conversion of testosterone to DHT, a more potent AR agonist. DHT acts specifically to masculinize the external genitalia of the male. Finasteride is a 5α-reductase inhibitor used clinically to combat androgen-dependent prostate cancer and more widely as a treatment for hair loss in adult men. Although this is not an environmental contaminant, it is used as an example because DHT plays such a significant role in the development of the male reproductive tract, and the impact of its inhibition on male reproductive development, especially the prostate and genitalia, is profound. Following oral exposure to rats on days 6–20 of gestation (Imperato-McGinley et al., 1992), AGD was reduced at doses as low as 0.003 mg/kg/day, hypospadias was observed beginning at 0.1 mg/kg/day, and 100% of the offspring were affected at 100 mg/kg/day. There was a significant decrease in prostate size at 25 and 50 mg/kg/day, with no further decrease at higher doses. Unlike AR blockade with flutamide, finasteride did not totally abolish prostate differentiation or completely feminize the external genitalia, despite increasingly higher doses. These results suggest that testosterone can compensate for DHT to some degree at the level of the AR. Wolffian differentiation, however, was not affected by inhibition of DHT, demonstrating its testosterone dependency, but seminal vesicle growth was impaired. AR blockade can inhibit testicular descent more effectively than inhibition of 5α-reductase activity (Spencer et al., 1991). It is suggested that finasteride causes hypospadias by preventing the formation of the medial mesenchymal plate that is necessary for assisting the movement of the urogenital sinus from the base to the tip of the genital tubercle (Clark et al., 1993). Additionally, external genital abnormalities can be produced in male rhesus monkey fetuses when dams are exposed to an oral dose (2 mg/kg/day) of finasteride on gestation days 20–100. No external genital malformations were seen in similarly exposed female fetuses or in fetuses of either sex following daily intravenous exposure of up to 800 ng/kg/day over the same period of gestation (Prahalada et al., 1997).

3.12.4.5 Phthalates. Phthalates are a broad class of chemicals used as plasticizers in a number of manufacturing processes, and as discussed below, the developmental effects of several phthalates (e.g., the dibutyl and diethylhexyl esters) are exerted via alterations in testosterone-synthesizing ability of the fetal testes. The reproductive toxicity in adults of some phthalates has been well described. For example, DEHP has been shown to target the rat testis of adults and juveniles (Gray and Butterworth, 1980; Sjoberg et al., 1985). The mode of action of the testicular toxicity is via a metabolite (the monoester, MEHP), with the target cell in the testis being the Sertoli cell, although the precise biochemical interaction has yet to be identified (Heindell and Chapin, 1989; Heindell and Powell, 1992). Attention has also been focused on the endocrine-active effects of phthalates, including interactions with both estrogen and androgen action. Zacharewski et al. (1998) reported that DBP, BBP, and DHP weakly competed with E_2 for binding to the ER in competitive ligand-binding assays. In gene expression assays using MCF-7 cells transiently transfected with Gal_4-HEGO, and the Gal_4-regulated luciferase reporter gene 17m5-G-Luc, 10 μM DBP, BBP, or DHP exhibited 36%, 42%, and 20% activity, respectively, when compared with the 100% response observed with 10 nM E_2. Only BBP induced luciferase activity (32%) in HeLa cells stably transfected with Gal_4-HEGO and 17m5-G-Luc constructs and imparted minimal ER-mediated viability to the E_2-dependent recombinant yeast strain PL3 on selective medium. No significant responses were observed with the five other phthalate esters in any of the *in vitro* assays. *In vivo*, none of the eight phthalate esters reproducibly induced significant increases in uterine wet weight in immature ovariectomized SD rats treated with oral doses of 20, 200, or 2,000 mg/kg of phthalate ester. Treatment with phthalate esters at the same doses did not affect the degree of vaginal epithelial cell cornification in mature ovariectomized rats. These results indicate that only selected phthalate esters (i.e., DBP, BBP, and DHP) exhibit weak ER-mediated activity in some *in vitro* assays at high concentrations, but none of the eight phthalate esters elicited *in vivo* estrogenic responses based upon results obtained from uterotrophic and vaginal cornification assays. These results serve to raise caution in assessing the potential hazard of chemicals based solely upon results of *in vitro* experiments.

More significantly, some phthalates (e.g., DEHP, DBP, BBP, di-isonyl phthalate, but not DEP, DMP or DOTP) induce antiandrogenic responses in fetal males. For example, male rat pups exposed during sexual differentiation to DBP or DEHP exhibit malformations in androgen-dependent tissues although apparently by a non-receptor-mediated mechanism (Gray et al., 1999a, 2000). Importantly, because the critical window lies outside that of the traditionally defined "period of organogenesis," these effects have been missed in standard developmental toxicology studies (Ema et al., 1992, 1993, 1994; Tyl et al., 1988; Narotsky et al., 1995).

A DBP multigenerational study showed marked effects on fertility of rats in the F_1 generation compared with their parents in the F_0 generation, with fewer and smaller litters and a 50% decrease in sperm count. In addition, these F_1 animals showed numerous male reproductive tract malformations at the highest dose level tested (~660 mg/kg/day) that were not observed at comparable dose levels in the standard developmental toxicity studies (Wine et al., 1997). Mylchreest et al. (1998) examined the critical differences in the exposure period between the F_0 and F_1 generations, and exposed pregnant and lactating animals and then examined their offspring. A high incidence of epididymal malformations and decreased sperm count were found, as well as delays in preputial separation and decreases in AGD of the male pups. All of the reproductive tract malformations seen in the multigeneration study could be reproduced in this shorter exposure regimen, and no effects were noted in female offspring. In another multigeneration study, however, exposure to 250 mg/kg/day DBP (the lowest dose tested) induced malformations in both male and female F_1 rats (Gray et al., 1999a). By narrowing the exposure window to just late gestation (gestation days 12–21), Mylchreest et al. (2000) essentially reproduced their earlier findings and also reported an increased incidence of retained nipples in the male offspring. Thus, DBP had all the attributes of a classical AR antagonist in affecting reproductive tract development with a LOAEL of 100 mg/kg/day that is much lower than LOAELs and most NOAELs for other toxicities of DBP. Mylchreest et al. (1999) compared the effects of DBP to the AR antagonist flutamide and showed many similarities in the pattern of effects but also a number of differences in tissue sensitivity, with the epididymis being the prime target for DBP malformations, whereas the prostate was the major target for flutamide. Neither DBP nor monobutylphthalate interacted directly with the AR.

That these effects are not mediated via AR antagonism are supported by the findings that DBP and DEHP, as well as their monoester metabolites, do not bind mammalian (rat or human) AR (Mylchreest et al., 1999; Parks et al., 2000). Although the precise cellular and molecular site of phthalate action in the fetal male is unknown, the testis appears to be the primary target (Mylchreest et al., 1998, 1999; Gray et al., 1999a; Parks et al., 2000). Maternal DEHP and DBP treatments induced dramatic reductions of fetal testosterone synthesis (Parks et al., 2000) and fetal androgen levels (Mylchreest et al., 1999; Parks et al., 2000), and altered Leydig cell morphology and function were evident. The fact that the selective AR for toxicity of the phthalates during development appears similar to that for testicular toxicity in pubertal males may suggest that some commonality in the initial molecular event initiates these adverse outcomes. It appears that the mechanism of action of phthalate-induced toxicity is widespread throughout vertebrates. Developmental reproductive toxicity of the phthalates is observed in the guinea pig, ferret (Lake et al., 1976), rabbit (DBP; Veeramachaneni, 2000), hamster (i.e., MEHP), and several strains of rats and mice, including the PPARα-knockout mouse. The fact that PPARα-knockouts display testicular and renal lesions after DEHP-treatment demonstrates that this receptor, apparently involved in the toxicity of MEHP in the liver, is not required for the expression of these other forms of toxicity (Ward et al., 1998).

A fish multigenerational study, which exposed medaka to DBP at environmentally relevant concentrations, detected abnormal gonadal function in the F_1 by not the F_0 generation (Patyna et al., 1999), although it failed to induce estrogenlike responses in this species. DBP also has been shown to alter androgen-dependent tissues in developing anurans (Higuchi et al., 1999; Ohtani et al., 2000).

3.12.5 AhR Agonists: TCDD, PCBs, and PCDFs

This class of EDCs is responsible for many well-characterized reproductive and populations effects in fish and wildlife (Peterson et al., 1993). Because these observed effects are used as support of the endocrine disruptor hypothesis and are believed to be caused by TCDD and structurally similar, synthetic halogenated hydrocarbons, it is important to understand the mechanism of action, as it relates to the endocrine disruptor hypothesis (Birnbaum, 1994). Although the effects caused by TCDD can be classified as an effect on signal transduction, these effects are not covered by the narrow definition of receptor-mediated effects of steroid hormones. Overall, the evidence supports the hypothesis that most, if not all, TCDD effects are mediated through the AhR (Okey et al., 1994; Hankinson, 1995), a cytosolic receptor protein that was first discovered by Poland and Glover (1977). The AhR signaling transcription pathway is initiated by TCDD diffusion into the cell, where it binds with high affinity to the cytosolic AhR protein complex, which also includes heat-shock protein 90 (Hsp90) and a 38-kDa, immunophilin-related protein (Ma and Whitlock, 1997; Carver and Bradfield, 1997). The ligand binding activates AhR and stimulates the dissociation of AhR-associated proteins. The ligand-receptor complex is subsequently translocated into the nucleus, where it dimerizes with AhR nuclear translocator (ARNT; Hankinson, 1995; Probst et al., 1993). The heterodimers are capable of recognizing and binding DNA at the consensus sequence, GCGTG, of dioxin-responsive elements (Denison et al., 1989; Dong et al., 1996). This action either increases or decreases the transcription of target genes (Nebert et al., 1993; Schmidt and Bradfield, 1996), including CYP450 (CYP1A1, CYP1A2; Quattrochi and Tukey, 1989), NAD(P)H:quinone reductase (Favreau and Pickett, 1991), class 3 aldehyde dehydrogenase (Asman et al., 1993), and glutathione *S*-transferase (Paulson et al., 1990).

The ARNT protein also pairs with HIF-1α to regulate genes active in response to low oxygen stress (Guillemin and Krasnow, 1997; Semenza, 1994; Wenger and Gassmann, 1997). Regulated genes include Epo for erythropoiesis (Semenza, 1994), VEGF for angiogenesis (Forsythe et al., 1996; Goldberg and Schneider, 1994; Maxwell et al., 1997; Shweiki et al., 1992), and GLUT-1 for glucose transport (Semenza et al., 1994; Wenger and Gassmann, 1997). AhR, ARNT, and HIF-1α belong to basic-helix-loop-helix/PAS protein family and are found in representative organisms of all five kingdoms (Hahn, 1998). In addition to responding to low oxygen in the case of ARNT-HIFα heterodimers, PAS proteins are involved in development and differentiation (Nambu et al., 1991; Isaac and Andrew, 1996), regulation of circadian clocks (Huang et al., 1995; King et al., 1997), and steroid receptor signaling (Yao et al., 1993). The fact that ARNT null mice are not viable beyond day 10.5 of gestation provides additional evidence of the importance of this protein (Kozak et al., 1997; Maltepe et al., 1997). It is possible that exposure to TCDD and subsequent recruitment of ARNT through AhR may inhibit other signal transduction pathways depending on ARNT (Chan et al., 1999). Thus, through its ability to interact with multiple signal transduction pathways and to induce or inhibit a variety of gene products, AhR agonists are capable of inducing a wide spectrum of biological effects at a number of different life stages and in a variety of species. Some of these responses do not easily fit the traditional definition of endocrine-mediated effects. In this assessment, biological effects that correlated with AhR occupancy were therefore not considered sufficient to invoke an endocrine-mediated

mode of action. Instead, the criteria listed at the end of this chapter were used to determine whether Ah-mediated effects would be included in this review. Because such information is less available for studies in wildlife, more leeway was used in determining whether to include findings in wildlife than in humans.

Male rats exposed *in utero* to a single dose of 0, 0.05, 0.20 or 0.80 µg/kg TCDD on day 15 of gestation displayed reduced fertility, as well as delayed puberty and altered reproductive organ weights (Gray et al., 1997a). Growth and viability of the pups were reduced only at 0.80 µg/kg, eye opening was accelerated (all dosage groups), and puberty was delayed (at 0.20 and 0.80 µg/kg). Treated progeny displayed transient reductions in ventral prostate and seminal vesicle weights, and epididymal sperm reserves and glans penis size were permanently reduced. Ejaculated sperm numbers were reduced (45% in the 0.8 and by 25% in the 0.05 and 0.2 µg/kg dosage groups) to a greater degree than were cauda or caput/corpus epididymal or testicular (unaffected) sperm numbers. Female offspring from this treatment regimen showed a delay in vaginal opening at 0.80 µg/kg. A persistent vaginal thread was present in 27% of the progeny at 0.20 and 92% at 0.80 µg TCDD/kg (Gray et al., 1997b). These effects did not appear to result from abnormal ovarian function during prepubertal development; neither serum E_2 levels nor ovarian E_2 production was reduced in 21- or 28-day-old progeny of dams exposed to 1 µg/kg. In addition, partial to complete clefting of the phallus was displayed in TCDD-treated rats (10% at 0.20 and 60% at 0.80 µg/kg), and these dosage levels also increased the length of the urethral slit, increased distance from the urethral opening to the tip of the phallus, and decreased distance from the urethral opening to the vaginal orifice. Although fertility rates were normal, time to pregnancy was delayed by treatment with 0.80 µg/kg. When necropsied at 20 months of age, females from the TCDD-dose groups displayed histopathological alterations of the reproductive tract. Thus, TCDD affects reproductive tract development in ways both similar and different than estrogens and antiandrogens. Fetal levels of TCDD as low as 8–13 ppt are associated with these reproductive alterations (Gray et al., 1995a, 1995b, 1997a, 1997b; Hurst et al., 1998, 2000a, 2000b).

Following exposure to a single dose of 2 µg/kg TCDD on gestation day 11.5, both control and TCDD-treated F_1 females mated successfully with a control male; 20% of the F_1 treated females did not become not pregnant (Wolf et al., 1999). In addition, 38% of pregnant F_1 females from the TCDD group died near-term, and there were reductions in the numbers of implants in pregnant animals and pups born live in the treated group. In the F_2, survival through weaning was drastically reduced (15% treated vs. 78% for control) by TCDD treatment of dams on postnatal day 0. F_1 female hamster offspring exposed *in utero* to TCDD also displayed external urogenital malformations, with most females having complete clefting of the phallus. Thus, adverse effects of TCDD persisted through two generations (F_1 and F_2), even though the F_1 generation was only indirectly exposed during gestation and lactation.

Altered neurological development is another health outcome associated with prenatal exposure to TCDD in experimental animals. Mably et al. (1992) reported demasculinization and feminization of sexual behavior in male rats following maternal exposure. At the age when sexual behavior was tested, AhR-dependent hepatic CYP450 levels and ethoxyresorutin dehydroxylase activity were not different from controls, thus demonstrating that effects on sexual behavior were due to long-lasting effects of developmental exposure and disturbance of organizational effects of sex steroids. In this model, levels of ERs in different brain regions were measured. (Bjerke et al., 1994). Although the AhR receptor is present in the developing nervous system, its role, if any, in normal development is unknown, and there is as yet no direct evidence for an implication of the AhR in brain development.

3.12.6 Mechanism for *p,p´*-DDE–Induced Eggshell Thinning in Oviparous Vertebrates

During the 1960s and 1970s, when the pesticide DDT was in the North American environment at greater concentrations, populations of several sensitive bird species declined because of unsuccessful incubation of eggs due to abnormally thin egg shells (Cooke, 1973). Many of these species (e.g., the double-crested cormorant) have experienced dramatic population increases since the use of DDT was banned in the United States and environmental concentrations have subsequently declined (Ludwig, 1984). The eggshell-thinning effect of *o,p´*-DDT and its potent, stable metabolite *p,p´*-DDE in sensitive species is well known. Species that normally produce eggs with a chalky valerite cover, including pelicans, cormorants, shags, and gannets, can produce eggs with a much reduced or completely absent cover following DDE exposure (Gould, 1972; Cooke et al., 1976). In these species, the shell-forming process was most impacted by DDE toward its termination. Other birds such as the great black-backed gull (Cooke, 1979a, 1979b) and the gray heron (Cooke et al., 1976) show a general reduction in all shell layers following DDE exposure. Changes in mineral composition of eggshells following DDE treatment have seldom been investigated (Longcore et al., 1971).

Several possible mechanisms of DDE-induced eggshell thinning (which may vary among species) have been suggested (Cooke, 1973, 1979a, 1979b). However, many of the most popular avian laboratory species, including the domestic chicken and the Japanese quail, are insensitive to DDE-induced eggshell thinning (Scott et al., 1975). Although the mechanism of eggshell thinning has never been completely deduced, research in this area has focused mainly on one sensitive avian species (Lundholm, 1980, 1982, 1984a, 1984b, 1984c, 1985, 1988, 1993, 1994; Lundholm and Mathson, 1983; Lundholm, 1987; Lundholm and Bartonek, 1991, 1992). Suggested mechanisms have included 1) limiting the supply of calcium to the shell gland from the blood by either changing uptake, excretion, or transport (Peakall et al., 1975; Haynes and Murad, 1985; Taylor and Dacke, 1984; Hagmann, 1982); 2) decreasing carbonate availability for shell formation via inhibition of carbonic anhydrase (Bitman et al., 1970; Peakall, 1970a, 1970b; Pocker et al., 1971; Cooke, 1973; Miller et al., 1976; Eastin and Spaziani, 1978); and 3) altering steroid hormone receptors or function (Lundholm, 1985, 1988).

Currently, the leading hypothesis regarding the mechanism of DDE-induced eggshell thinning involves an inhibition of PGs by the shell gland mucosa. PGs play an important role in the control and regulation of reproduction in birds (Lundholm and Bartonek, 1992). PG synthesis is decreased by *p,p´*-DDE in duck shell gland mucosa, both in *in vitro* experiments and following *in vivo* exposure (Lundholm and Bartonek, 1992). PG synthesis was not inhibited by *p,p´*-DDT or *o,p´*-DDE in *in vitro* experiments, in keeping with the potency of these congeners in causing egg shell thinning. Additionally, indomethacin treatment reduced eggshell thickness. It has been hypothesized that a furosemide-insensitive, PG-stimulated HCO_3^- transport could be inhibited in the shell gland mucosa of DDE-treated ducks, but further experiments have not supported that hypothesis (Lundholm, 1994).

The mechanism of DDE-induced eggshell thinning has been suggested to be quite complex. Eggshell thinning is associated with a decreased quantity of calcium in affected eggs, and in the mallard duck the effect of DDE has been associated with a decreased transport of calcium from the eggshell gland mucosa to the lumen fluid. Treatment of birds with DDE has been associated with a variety of biochemical changes that could be related to changes in calcium transport. Many of these biochemical end points are interrelated, and it is difficult to determine which are the direct targets of DDE and which are merely co-influenced by its action. The situation is complicated by the fact that sensitivities to DDE-induced eggshell thinning vary among avian species, and hence different mechanisms might be causing eggshell thinning in different species, as evidenced by different gross eggshell defects.

Although egg shell thinning induced by DDE and related chemicals is one of the most cited examples of endocrine disruption in wildlife, given the multiple hypothesis regarding the mode of action, it cannot be stated with certainty that it is indeed an result of endocrine disruption. The strongest evidence for the linkage comes from the findings of altered PG biosynthesis in the mucosal gland.

3.13 EDC Modes of Action for Carcinogenesis—The Effect of Atrazine

Concern for the endocrine-disrupting effects of atrazine, a triazine herbicide, arose following the observation of increased incidence of mammary tumors in a chronic bioassay in female SD rats exposed to 400 ppm atrazine in the diet for 104 weeks. These tumors also appeared in control females but occurred earlier in the treated females. No other tumors were present in the treated SD female rats or in male SD rats or male and female Fischer 344 rats (Stevens et al., 1994; Thakur et al., 1998).

The finding of earlier-onset mammary tumors led to an investigation into the estrogenicity of atrazine, but (under equilibrium conditions) atrazine was not able to compete with E_2 for binding to rat uterine ERs. A weak competition was noted if the cytosols were preincubated at 25°C prior to incubation with the tracer (Tennant et al., 1994a). Somewhat conflicting results have been seen in other studies. Daily exposure of adult Fischer rats to 120 mg/kg for 7 days resulted in fewer treated females displaying normal estrous cycles, and the number of days in diestrous was significantly increased. Fertility was reduced in females during the first week after exposure, but pregnancy outcome was not affected in those that became inseminated (Simic et al., 1994). However, treatment of adult, ovariectomized SD rats with up to 300 mg/kg atrazine by oral gavage for 3 days did not result in an increase in uterine weight, nor were there increases in uterine progesterone levels, suggesting the lack of an estrogenic potential. When E_2 (2 µg/kg s.c.) was given in conjunction with 300 mg/kg or orally administered atrazine, there was a weak inhibition (~25%) of the uterotrophic response (Tennant et al., 1994b). In a similar study, immature female SD rats were dosed with 0, 50, 150, or 300 mg/kg atrazine by gavage for 3 days. Uterine weight was not increased, but decreases in uterine progesterone receptors and peroxidase activities were noted; however, when combined with E_2, no antiestrogenic effect of atrazine was noted on the uterus, including decreases in uterine progesterone receptor binding and uterine peroxidase (Connor et al., 1996). In this same study, atrazine did not affect basal or E_2-induced MCF-7 cell proliferation or display agonist or antagonist action against E_2-induced luciferase activity in MCF-7 cells transfected with Gal_4-regulated human ER chimera.

To further evaluate effects on reproductive function, female LE and SD rats that had been screened for regular 4-day estrous cycles received 0, 75, 150, or 300 mg/kg/day atrazine by gavage for 21 days. In both strains, atrazine disrupted the regular 4-day estrous cycles. For the LE rats, all dose levels were effective, whereas SD rats required a higher dose (150 mg/kg/day) for a longer time for this effect to appear. The increased time spent in vaginal diestrus was associated with elevated serum progesterone and low E_2 concentrations, indicative of a repetitive pseudopregnant condition. This hormonal condition was not considered by the authors to be conducive to the development of mammary tumors, although there was some indication of prolonged estrous at the lowest dose tested (Cooper et al., 1996).

The strain difference noted in the premature onset of mammary tumors (insensitive Fischer 344 rats vs. sensitive SD rats) has been attributed to differences in the normal aging of the reproductive tract in these strains (Eldridge et al., 1994; Stevens, et al., 1994; summarized in Chapin et al., 1996). Reproductive cycling in the female SD rat begins to decline in animals less than 1 year of age, presumably due to the loss of sensitivity of adrenergic neurons in the hypothalamus that control GnRH release to the pituitary. This loss of stimulation reduces FSH and LH release and ultimately delays ovulation. The delayed ovulation, in turn, allows prolonged exposure to estrogens and an effect evident as persistent vaginal cornification. In contrast, adrenergic neurons of female Fischer 344 rats do not seem to lose their sensitivity to estrogen stimulation, and regular cycling is maintained for a much longer time period. Rather, reproductive aging in the Fischer 344 is believed due to inability to control daily PRL surges, a prolonged activity of the corpora lutea, and a higher level of progesterone release. Hence, the endocrine milieu of the aging SD rat, but not the Fischer 344 rat, favors development of mammary tumors and helps explain the difference in incidence of spontaneous tumors as females of these strains age.

Consistent with an effect on central nervous system function, atrazine exposure beginning at weaning alters the development of puberty in both the male (Stoker et al., 2000) and female rat (Laws et al., 1996; 2000b). In the male, doses as low as 12.5 mg/kg/day beginning on postnatal day 23 delayed preputial separation. At postnatal day 53, ventral prostate weights, but not testes weights, were reduced in rats treated with 50 mg/kg/day. Female rats were somewhat less sensitive, as it required 50 mg/kg/day to delay vaginal opening, and 100 mg/kg/day altered estrous cycles in the first 15 days after vaginal opening. In addition, *in vitro* studies involving PC12 cells have suggested that atrazine inhibits the cellular synthesis of dopamine mediated by tyrosine hydroxylase, and norepinephrine mediated by dopamine β-hydroxylase and, as result, reduces the potential of the neuronlike cells to release norepinephrine (Das et al., 2000). How atrazine accelerates the neuroendocrine aging of the reproductive axis in the SD rat, however, has not been determined.

Atrazine may exert neuroendocrine effects in other vertebrates. Ovulated female Atlantic salmon (*Salmo salar*) release a priming pheromone in the urine (an F-type prostagladin) that is subsequently detected by the olfactory system of the mature male salmon and results in increased levels of sex steroids and expressible milt. Short exposure of atrazine to male parr significantly reduced the olfactory response to PG F_{2a}. In addition, similar exposures reduced their ability to respond to the priming effect of ovulated female salmon urine. Atrazine also had an additional effect upon the testes, modifying the release of androgens and suggestive of an additional mode of action in this species (Moore and Waring, 1998).

3.14 EDC-Related Modes of Action in Neurotoxicity

3.14.1 Overview

There is evidence of neurotoxicity for over 850 workplace chemicals (IPCS, 2001b), including metals, organic solvents, agrochemicals, polyhalogenated aromatic hydrocarbons, natural neurotoxins, and pharmaceuticals/drugs of abuse. Because the reproductive endocrine system is primarily regulated by the neuroendocrine system, these chemicals are potentially EDCs. However, even with the close interactions between the nervous and endocrine systems, it has generally proven difficult to elucidate primary modes of action from secondary manifestations, even for chemicals that have known potential to influence hormone action. Although the mechanisms by which endocrine disruptors influence the nervous system are largely unknown, it is clear that two different modes of interaction of hormones with neural function must be considered: 1) effects related to activational properties of hormones in adult organisms resulting in transient changes and 2) organizational effects on hormone-dependent processes during neural development that can result in permanent changes of neurobehavioral function, particularly sex-dependent and sexual-related behaviors. Both actions may involve specific hormone receptors, such as the estrogen or the AR, or may be due to modulations of receptors for neurotransmitters that are reported to be influenced by hormones. For instance, GABA receptors, muscarinic and nicotinic receptors, NMDA receptors, σ-receptors, and neuropeptide receptors are implicated in steroid hormone action as well as membrane receptors coupled to second messengers (Mensah-Nyagan et al., 1999).

To further complicate matters, the nature of the influence on neurotransmitters and whether it is endocrine mediated may differ even with compounds that are structurally closely related. For example, prenatal/neonatal exposure to the 3,4,3′,4′-tetrachlorobiphenyl resulted in elevated concentrations of dopamine in the frontal cortex and of dopamine and its metabolites in the substantia nigra, whereas exposure to 2,4,2′,4′-tetrachlorobiphenyl resulted in significant decreases in concentrations of dopamine in the frontal cortex and caudate nucleus. In both cases, the changes persisted into adulthood (Seegal et al., 1997). The study suggested that the reductions in brain dopamine concentrations were a consequence of PCB congener–induced inhibition of the synthesis of dopamine in concert with changes in cholinergic receptor function, whereas the persistent elevations in brain dopamine may be mediated by alterations in steroid hormone function during key developmental periods. Coplanar congeners, in addition to their ability to interact at the AhR, also alter estrogenic function, either by enhancing the metabolism of estrogens to hydroxy- and catecholestrogens (Gierthy et al., 1988) or by down-regulating ERs (Safe et al., 1991).

Although chemicals that alter neurotransmitter concentrations, such as PCBs, are likely to influence neuroendocrine function and ultimately reproduction, there are only a few reports on this potentially important mechanism of endocrine disruption. Reproductive impairment in Atlantic croaker exposed to Aroclor 1254 was associated with a dramatic decline in LH secretion and hypothalamic levels of 5-HT, a neurotransmitter that has a stimulatory influence on LH secretion (Khan and Thomas, 1998). A subsequent study showed that that the decline in 5-HT concentrations was due to inhibition of tryptophan hydroxylase, the rate-limiting enzyme in 5-HT synthesis (Khan and Thomas, 2001). The decreased 5-HT activity after PCB exposure resulted in decreased hypothalamic concentrations of LHRH and its secretion, leading to the down-regulation of LHRH receptors on gonadotropes and a decreased LH response to LHRH stimulation (Kahn and Thomas, 2000). Moreover, a specific inhibitor of tryptophan hydroxylase, parachlorine phenylanaline, mimicked these neuroendocrine effects of the PCB mixture as well as the subsequent reproductive impairment, whereas cotreatment of the PCB-dosed fish with 5-hydroxytryptophane, which bypasses this biosynthetic step, reversed the PCB effects (Khan and Thomas, 2001). Neuroendocrine disruption associated with alterations of neurotransmitter function has also been reported in croaker after exposure to lead (Thomas and Khan, 1997).

The distinction between chemical exposures of the developing as opposed to the mature nervous system is of particular importance in the present context for both toxicological and neurobiological reasons. Both the nature and the adversity of outcome may depend on the time window during which chemical exposure occurs. Some hormones, such as sex steroids or thyroid hormones, are known to have a strong and strictly time-coupled organizational impact on brain development (Gray and Ostby, 1998). From a neurobiological point of view, disruption of organizational factors during development in general and, more specifically, during brain development is important because long-lasting or irreversible neurobehavioral changes later in life may be the consequence of such interactions (Tilson, 1998). For example, thyroid hormones are known to affect brain development a) by increasing the rate of neuronal proliferation in the cerebellum, b) by timing neuronal proliferation and differentiation, and c) by organizing the pattern of neuronal migration to specific brain areas (Porterfield, 1994). In humans, endemic cretinism caused by iodine deficiency, congenital hypothyroidism, or maternal hypothyroidism is associated with well-known neurological and behavioral deficiencies, such as mental retardation, deaf-mutism, speech disorders, or motor deficits (Porterfield, 1994). However, the degree of thyroid dysfunction is clearly critical.

In the adult organism, endocrine disruption of the reproductive system is considered as a possible cause of neurobehavioral alterations either if gonadal hormones are shown to be affected in association with changes in reproductive behavior and nonreproductive neurobehavior, or if sexually dimorphic nonreproductive behavioral changes following chemical exposure are reported without endocrine data. A direct toxic effect on hormone-receptor-expressing neurons and glial cells must also be considered. Alterations of hormone concentrations may be caused by cytotoxic effects on hormone-producing organs, resulting in impaired synthesis or release of hormones. For example, PCB exposure resulted in fine structural lesions in the thyroid and inhibition of proteolysis of thyroglobulin, thereby decreasing the release of T_4 (Collins and Capen, 1980). In addition, thyroid hormone levels are influenced by increase in hormone metabolism due to PCB exposure (Barter and Klaassen, 1992) and by blocking the binding sites for T_4 at its serum transport proteins, which causes enhanced clearance from serum and decreased availability to tissues (Brouwer and Van den Berg, 1986).

The question of which mechanism underlies the effect of putative endocrine disruptors on a given neurobehavioral function ultimately depends on what is known about the regulation of that function at the cellular and subcellular level. However, the events that constitute the basis for higher neural functions are far from being understood for many of the end points investigated. The following are two examples of experimental studies in which attempts were made to delineate possible mechanisms for chemical-induced alterations of neuroendocrine and neurobehavioral effects. However, these studies are only the first steps in the elucidation of the mechanisms.

3.14.2 Sexual Differentiation of the Nervous System

It is generally assumed that sexual differentiation of the brain in rodents depends on the activity of aromatase (CYP19), an enzyme that converts androgens to estrogens. Aromatase has been detected in all mammal brains so far examined (Lephart, 1996), but its role in sexual differentiation in other than rodent species remains to be proven. Aromatase is expressed in several brain areas, including the HPOA, stria terminalis, amygdala, and striatum. Its regulation appears to differ in different brain areas regarding androgen dependence and the developmental phase of maximum activity (Lephart, 1996; Lauber et al., 1997a, 1997b; Küppers and Beyer, 1998; Roselli et al., 1998). In the HPOA, a region with several sexually dimorphic nuclei (Cooke et al., 1998), a sharp peak of activity was found at the end of gestation that declines to basal activity within the first 5 days after birth (Lephart, 1996).

Maternal exposure to a mixture of PCBs, reconstituted according to the congener pattern found in breast milk and containing *ortho*-chlorinated and coplanar congeners, caused decreases in aromatase activity at birth of male rat pups together with an elevated sweet preference and reduced testes weights and testosterone levels in adult male offspring (Hany et al., 1999). Sweet preference behavior is more pronounced in female rats, suggesting that reductions in hypothalamic E_2 results in a more femalelike differentiation of the brain that in turn causes a feminization of behavior in adulthood.

3.15 EDC-Related Modes of Action in Immunotoxicity

The major function of the immune system is to defend against infectious agents and certain neoplastic cells. Various cell types and their soluble mediators execute the function of the immune system in finely tuned concert. The maintenance of homeostasis requires bidirectional communication between the neuroendocrine and immune systems. Most of the influence of the brain on the immune system is exerted by hormones released by the neuroendocrine system. Indeed, receptors for hormones have been detected on cells of the immune system, whereas receptors for cytokines have been detected in the endocrine glands and brain. It is also noteworthy that almost all lymphoid tissues are innervated, although the role of this neuroregulatory pathway is largely unknown (reviewed by Heijnen et al., 1991; Weigent and Blalock, 1995; Besedovsky and Del Rey, 1996; Johnson et al., 1997).

The HPA axis represents the major pathway in the communication between the central nervous system and the immune system. Synthesis of glucocorticosteroid hormone (cortisol in man) by the adrenal gland, induced by ACTH from the pituitary gland, results in suppression of immune responses. Other mechanisms are those mediated by the direct action of neuropeptides, such as opioid peptides (Van den Berg et al., 1991), on immune cells that are either stimulatory or inhibitory. For their communication, cells of the immune system carry receptors for a number of hormones, neuropeptides, and neurotransmitters, such as CRH, ACTH, PRL, β-endorphin, GH, and sex steroids. In addition, cells of the immune system produce inflammatory cytokines, specifically, tumor necrosis factor-α, IL-1, and IL-6, that may act as endocrine hormones of the immune system, produced at distant sites and acting upon the central components of the HPA axis and the sympathetic system.

Cortisol, the final effector of the HPA axis, has multiple and profound immunosuppressive effects. Histologically, the thymus is the first organ affected by this hormone. Cortisol affects production, traffic, and function of leukocytes; this often leads to lymphopenia and monocytopenia. In addition, monocyte chemotaxis, bactericidal activity, and T-lymphocyte proliferation can be inhibited by cortisol. Glucocorticoids also inhibit the production of many cytokines. In addition, glucocorticoids inhibit the expression of adhesion and adhesion receptor molecules on the surface of immune and other cells and potentiate the acute-phase reaction induced by cytokines, primarily IL-6.

PRL has been shown to regulate various aspects of the immune system. Hypoprolactinemia is associated with impaired lymphocyte proliferation and decreased production of macrophage-activating factors by T lymphocytes. The endogenous opioid peptides α-endorphin, β-endorphin, and γ-enkephalin are also produced in the pituitary gland. Endorphin receptors similar to those in the brain are present on spleen cells and probably several others types of leukocytes. β-Endorphin has been shown to enhance T-cell proliferation and IL-2 production. One biological activity of the thymus that is under neuroendocrine control is the secretion of thymic hormones (Savino and Arzt, 1999). The secretion of thymulin, a nonapeptide produced by thymic epithelial cells, is modulated by GH and PRL. The interaction between the pituitary and thymus is demonstrated by the immunodeficiency of the thymus-dependent immunity that occurs in mice following injection with antisomatotrope hormone serum.

Modifying influences on immune responses have also been reported for sex steroids. The balance between male and female sex hormones, E_2 and testosterone, influences the extent of immune responsiveness. In general, the male sex hormone testosterone is immunostimulatory. E_2 and synthetic nonsteroidal estrogenic compounds such as DES are potent suppressors of specific immunity: the effects observed in rodents include thymic atrophy, suppression of thymus-dependent cellular immune responses, acceleration of autoimmune diseases, suppression of natural killer cell activity, myelotoxicity, and stimulation of the mononuclear phagocyte system (Luster et al., 1984). Considerable changes are seen in lymphoid organs during pregnancy, and increased serum E_2 levels during pregnancy correlate with lymphopenia and suppression of cellular immunity (Clarke, 1984). By evaluation of steroidal and nonsteroidal compounds with varying degrees of estrogenicity, Luster et al. (1984) provided evidence that immunotoxicity correlated for the most part with estrogenicity.

In humans, both the neuroendocrine system and the immune system are immature at birth and fully develop at later stages in life. The immune system is highly sensitive to regulation by glucocorticoids in the human newborn, a period of life in which the capacity to generate a cortisol response is decreased, suggesting that this represents an adaptational response of the immune system that preserves the important regulatory effects of glucocorticoids on the immune system during this delicate developmental period (Kavelaars et al., 1996). This may imply that the neuroendocrine system may also be very sensitive to EDCs during ontogeny. Regarding the immune system, its susceptibility to toxic compounds is most evident during the perinatal period of life, as shown in laboratory animal studies with various compounds, including TCDD (Vos and Moore, 1974) and hexachlorobenzene (Michielsen et al., 1999).

These considerations show that there are potentially multiple endocrine pathways that may influence the function of the developing and mature immune system and that these may be targets for EDCs.

3.16 Basis for Attribution of Effects to Endocrine Disruption

The foregoing examples were cases in which the mode of action of an EDC has been generally well characterized in the laboratory setting in order to illustrate the types of interferences that are of importance and the variety of manifestations of adverse health outcomes that can occur. Some of the principles for defining cause-and-effects relationships that emerge from these examples, and that are appropriate to consider in the context of the rest of this review, are as follows:

(1) Ability to isolate the response to endocrine sensitive tissues in an intact whole organism
(2) Analysis of the response at multiple levels of biological organization—from phenotypic expression to physiology, cell biology, and ultimately molecular biology
(3) Direct measurement of altered hormone action (gene induction or repression), hormone release, hormone metabolism, or hormone interactions under the experimental regime in which the toxicologic outcome was manifest
(4) Dose–response observations that indicate the perturbance of the endocrine system is a critical response of the organism, and not the secondary result of general systemic toxicity
(5) Ability to compare resulting phenotypes with outcomes from exposures to known pharmacological manipulations
(6) Indication that there is differential sensitivity of certain life stages in which dysregulation of a particular endocrine system is known to have adverse health consequences
(7) Ability to restore the phenotype or toxicologic outcome via pharmacological manipulations that counter the presumed mode of action on the endocrine system in the intact organism
(8) Supporting data on endocrine activity from *in vitro* binding, transcriptional activation, or cellular response studies

Clearly, not all these components need be present in a single situation for a determination to be made that a chemical exposure has had an adverse health impact via modification of the normal functioning of the endocrine system. However, a collective weight-of-evidence approach is needed to classify the conditions under which the exposure is "endocrine disruptive." In Chapter 7 of this assessment, we extend the evaluation of cause and effect to natural populations, both human and wildlife, with particular emphasis on the presumed mechanistic basis for which a particular outcome is linked to particular exposure.

Considerable progress has been made in identifying unique life history traits that may increase the risk of EDC exposure to wildlife compared with traditional laboratory animal models or humans. In fact, consideration that wildlife may be uniquely vulnerable to certain EDCs is justified for several reasons. It is conceivable that chemicals not identified as EDCs based on the responses of laboratory animal models may affect the endocrine systems of species that differ considerably. Also, certain aspects of the life history of a species may be uniquely sensitive to EDCs (critical periods of development). Other aspects of their life history that differ either qualitatively or quantitatively from other animals may predispose certain species through avenues (food source, habitat contamination levels) that increase their risk of chemical exposures.

There are aspects of the endocrinology of vertebrates, such as key hormonally regulated events involved in reproduction and development, that show little difference between wildlife species and animal models or humans (Norris, 1996; Ankley et al., 1998a; Van Der Kraak et al., 1998a). As of yet, and perhaps not surprisingly, research efforts have yielded inconsequential evidence to support the idea that vertebrate wildlife are inherently more sensitive to EDCs than are laboratory animal models or humans. However, despite recent progress made in the understanding of the endocrine systems of some wildlife species, conclusions in this regard are hindered by current knowledge gaps of the biological processes of many wildlife species in question.

Vertebrate wildlife species are not the only animals that may be adversely affected by EDCs. The potential risks of EDCs to invertebrates warrant special consideration given the highly divergent nature of endocrine systems within these taxa, which also differ markedly compared with vertebrates (LeBlanc et al., 1999). For example, invertebrate endocrine systems operate with different proteins, as well as hormones unique to these taxa, such as terpenoids and ecdysteroids (ecdysone, 20-hydroxyecdysone). Homoses-quiterpenoid epoxides and methyl farnesoate, which serve as juvenile hormones in insects and crustaceans, respectively, are other examples of hormones not found in vertebrates (Cymborowski, 1992; Laufer et al., 1993). These obvious differences make it apparent that responses to environmental chemicals in invertebrates cannot be predicted based on the endocrine responses of traditional laboratory animal models or humans.

One aspect of life history that is common to a wide array of wildlife species and not displayed in common animal models is oviparity. Oviparous species require unique consideration in terms of exposure during early development. An extensive review of the consequences of oviparity in the context of exposure characterization for EDCs was developed by Kleinow et al. (1999); only a few of the more salient points of their analysis are covered here. First, as opposed to viviparous species, the chemical dose to which early developmental stages of oviparous animals are exposed is derived largely via maternal transfer associated with deposition of nutrients to the maturing oocytes. Therefore, although the egg affords some degree of protection from contaminants in the external environment, it also can subject embryos to elevated, maternally derived, chemical exposures during potentially sensitive early developmental stages. A first-order approximation of the initial exposure received by oviparous embryos can be obtained by assuming a lipid-normalized relationship between contaminants in the maternal organism versus those in her eggs (Russell et al., 1999). However, this relationship has only been characterized for relatively stable, nonionic organic chemicals. Furthermore, the closed environment of the egg (i.e., little excretion) and the minimal biotransformation abilities of young embryos create unpredictable exposure variations (in terms of toxicokinetics) as the animals develop. Contaminants also partition from compartments associated with nutrient storage to developing tissues. The degree to which oviparity is an important and/or unique variable with respect to predicting EDC effects will depend upon the physicochemical characteristics of the contaminant of concern, and the mechanism(s) of action under consideration.

Other aspects of life history that differ in wildlife species may complicate efforts to generalize the potential impacts of EDCs across species. For example, there are certain developmental events that are unique to specific species or classes of vertebrates such as external metamorphosis in amphibians, marine flatfish, and agnathans (lamprey), or smoltification in salmonids. In addition, processes unique to some taxa of invertebrates (i.e., molting, limb regeneration, diapause, pheromone production, pigmentation, metamorphosis) are also under endocrine control. In general, wildlife studies have not considered the implications of timing of exposure to EDCs relative to these critical aspects of reproduction or development.

This chapter evaluates the extent to which wildlife species, including both vertebrates and invertebrates, have been affected by environmental compounds whose primary mode of action involves endocrine disruption. This includes a consideration of the unique aspects that make the various taxa susceptible to the potential effects of EDCs. Subsequently, field observations and supporting laboratory data are critically evaluated with respect to the plausibility that responses are linked to exposure to substances that function as EDCs. The intention here is not to provide an extensive review of all examples of alterations in reproduction and development that have been attributed to chemical exposures. Rather, the approach is to

List of Abbreviations

11-KT 11-Ketotestosterone
17α,20β-P 17α,20β-dihydroxy-4-pregnen-3-one
ACTH Adrenocorticotropin hormone
AhR Aryl hydrocarbon receptor
BKME Bleached Kraft mill effluent
DDD Tetrachlorodiphenylethane
DDE Dichlorodiphenyl dichloroethylene
DDT Dichlorodiphenyl trichloroethane
E_2 17β-Estradiol
EDCs Endocrine-disrupting chemicals
EMS Early mortality syndrome
HPI Hypothalamic-pituitary-interrenal
NP Nonylphenol
NRC National Research Council
OCs Organochlorines
PAH Polyaromatic hydrocarbons
PCBs Polychlorinated biphenyls
PCDDs Polychlorinated dibenzodioxins
PCDFs Polychlorinated dibenzofurans
PPR Precloacal length to the posterior lobe of the plastron
RA Retinoic acid
STW Sewage treatment works
TBT Tributyl tin
TCDD 2,3,7,8-Tetrachlorodibenzyl-*p*-dioxin
TSD Temperature sex determination
UNEP United Nations Environment Programme
USA United States of America
VTG Vitellogenin

focus on selected cases studies that are sufficiently developed that they may ultimately be evaluated using a weight of evidence approach (Hill, 1965; Fox, 1991; Ankley and Giesy, 1998a). Finally, some of the uncertainties and data gaps that confound our understanding of the extent to which wildlife species have been and may continue to be affected by exposure to EDCs are considered.

4.1 Mammals

4.1.1 Unique Aspects

Current-day mammals are represented by over 4,500 species, so it is not surprising that they possess a wide array of life history strategies (Vaughan et al., 2000). In monotremes, represented only by the duck-billed platypus and the echidna, females lay leathery-shelled eggs like those of turtles. In marsupials, such as kangaroos, wombats, and opossums, embryos develop in the uterus until they have enough musculature and coordination to move by themselves. The young then leave the uterus, crawl into the mother's pouch, and continue their development attached to one of her mammary glands. However, the vast majority of mammalian species have young that develop inside the uterus attached to a placenta. Because all species of mammals suckle their young, this represents an important avenue for exposure to chemical contaminants during critical periods of development.

Fish-eating mammals may be particularly vulnerable to EDCs because of (1) their position in the food chain, (2) dependence on an aquatic/marine foodweb rather than a terrestrial foodweb, (3) occurrence in areas influenced by industry and agriculture, and (4) specific aspects of their reproductive physiology. As high trophic consumers, these species often acquire large burdens of persistent pollutants through the biomagnification of compounds obtained from contaminated prey, including many potential EDCs (Tanabe et al., 1988). Pinnipeds and cetaceans have relatively large amounts of blubber for insulation and readily bioaccumulate highly persistent OC insecticides, PCBs, and other lipid-soluble compounds. Indeed, both reproductive and nonreproductive toxicity has been found in many fish-eating mammalian species that live in riverine or coastal areas where contaminant burdens are generally higher than in more open ocean or pelagic systems. Some of the best evidence for a relationship between OCs and reproductive toxicity stems from field studies on seals (Bergman and Olsson, 1985; Reijnders, 1986) and mustelids (Wren, 1991; Kihlström et al., 1992; Leonards, 1997). These species exhibit the phenomenon of embryonic diapause, which may be a critical phase in the reproductive cycle that is prone to disruption by contaminants (Reijnders and Brasseur, 1992).

Feeding strategy may directly impact exposure to EDCs. For example, studies by Talmage and Walton (1991), which compared the contaminant burdens of rodents, demonstrated that insectivores (shrews) had the highest contaminant burden, followed by omnivores (cricetid mice), and finally herbivores (voles). But herbivores may be uniquely exposed to plant-derived compounds that affect the endocrine system. For example, the steroid alkaloid plant toxins (jervine, 11-deoxyjervine, and 3-*O*-glucosyl 11-deoxyjervine) were demonstrated to cause teratogenesis in sheep grazing on the range plant *Veratrum californicum* in western North America (Omnell et al., 1990; Cooper et al., 1998). There are numerous other examples of plant-derived compounds that alter various aspects of development (Keeler and Panter, 1989; Bunch et al., 1992). However, establishing whether or not these compounds affect endocrine function and, if so, whether these effects manifest in wildlife requires further investigation.

4.1.2 Effect-Based Responses and Case Studies

4.1.2.1 Reproductive dysfunction in mustelids. Populations of mustelids, including mink (*Mustela vison*) and Canadian otter (*Lutra canadensis*), have declined in regions of the Great Lakes, where these species have a high content of fish in their diet (Wren, 1991). Because Great Lakes' fish contain elevated concentrations of numerous synthetic OCs including pesticides and PCBs, it is plausible to hypothesize a contaminant-based etiology. Several studies have shown that ranched mink fed a diet of Great Lakes' fishes, predominantly salmonids, show adverse reproductive outcomes (Ankley et al., 1997). Of all the pollutants that Great Lakes' mink may be exposed to, PCBs and, to a lesser extent, TCDD have the greatest impact (Giesy et al., 1994a). Yet, data are not available that would provide conclusive evidence that these compounds are linked to effects at the population level of wild mink. In particular, data that link wild mink exposed to persistent OCs, such as PCBs, PCDFs, and PCDDs, and changes in population dynamics or endocrine dysfunctions are missing. In the absence of an understanding of the underlying mechanisms mediating these effects, the link between these compounds and endocrine function is tentative at best.

A recent study using mink demonstrated that exposure to low doses of PCBs over 18 months, including two reproductive seasons, impaired reproduction (Brunstrom et al., 2001). The observed effects included fetal deaths, abnormalities, decreased kit survival, and decreased kit growth. The authors state that the doses of Clophen A50 (a technical PCB preparation) given are relevant in terms of wildlife PCB exposure via the diet in contaminated areas. Furthermore, the reproductive dysfunction was found to involve PCB congeners that are AhR agonists (Brunstrom et al., 2001).

Further evidence that mustelids may be sensitive to environmental compounds comes from a study of river otter on the Columbia River in the northwestern United States. In this case, there were reports of reduced baculum length and weight and no evidence of spermatogenesis in 8–10-month-old individuals (Henny et al., 1996). These effects seem to result from delayed development and are transient, as they do not persist into adulthood. These responses were correlated with a number of OC insecticides, PCBs, dioxins, and furans. In addition, European otter (*Lutra lutra*) populations in Europe declined dramatically during the 1960s to 1980s, and exposure to PCBs that result in impaired reproduction has been considered to be a major cause of this decline (Kihlström et al., 1992; Leonards, 1997; Brunstrom et al., 1998; Roos et al., 2001). A recent study by Roos et al. (2001) on Swedish otter populations concluded that these populations have increased during the 1990s. This improvement coincides with a decrease of PCB concentrations in the Swedish environment; supporting the idea that PCB contamination has been the lead cause of the declines of European otter populations in the past decades (Roos et al., 2001). However, at this time, there is inadequate population data providing a link between exposure and reproductive outcome and insufficient knowledge on the impact of other environmental stresses.

4.1.2.2 Reproductive dysfunction in marine mammals. Harbor seals (*Phoca vitulina*) in the Dutch Wadden Sea exhibited low reproductive success and declining population numbers that were attributed to the impact of PCBs (Reijnders, 1980). Comparative population studies showed that the observed population decline (66%) could be fully accounted for by the observed decreased reproduction. Other studies showed that female harbor seals fed fish from the polluted Wadden Sea exhibited a lower reproductive success (50%) than did seals fed less

contaminated fish from the Atlantic. In the same study, implantation failure was found to be associated with lower levels of E_2 (Reijnders, 1986; Reijnders, 1990). Despite the strong correlation with OC exposure, there is still an incomplete understanding of the specific compounds responsible for the pathological effects and their mechanism of action(s) (Troisi and Mason, 1998; Reijnders, 1999). It is plausible that PCB-induced reductions in E_2 levels are the result of alterations in enzyme metabolism (Reijnders, 1999). This hypothesis is based on the observation that induction of the P450 isoenzyme CYP1A(2) causes increased hydroxylation of E_2 (Funae and Imaoka, 1993), combined with the findings that CYP1A(2) is significantly induced in PCB-exposed harbor seals (Boon et al., 1987). More detailed studies are required to substantiate this hypothesis.

DeLong et al. (1973) found stillbirths and premature pupping in California sea lions (*Zalophus californiansus*) to be associated with high PCB and DDE levels. Further research revealed that diseases (leptospirosis and calcivirus infections) were prevalent in the sea lions that could also contribute to abortions, endocrine disorders, and premature pupping (Gilmartin et al., 1976). Because both the EDCs and the diseases could have the same effect, it is not possible to discern a cause-and-effect relationship between contaminant levels and effects on reproduction. More studies are needed to clarify the underlying mechanisms.

There is ample evidence that populations of Baltic ringed seals (*Phoca hispida botnica*) and gray seals (*Halichoerus grypus*) have declined markedly over the past 100 years (Helle, 1983; Bergman and Olsson 1985; ICES, 1992). Although overhunting and habitat destruction may have been contributing factors, it is generally accepted that persistent pollutants, which adversely affected the reproductive performance of the females, resulted in the decline in seal numbers. Some of the disorders observed in exposed seals include interruptions during early pregnancy, stenosis and occlusions, and partial or complete sterility of Baltic ringed seals (70%) and gray seals (30%). A time trend study by Roos et al. (1998) examined PCB and DDT concentrations in 177 juvenile gray seals collected between 1969 and 1997 along the Swedish Baltic coast. Their results also implicate PCBs as the leading cause of the reproductive dysfunctions observed in Baltic gray seals. Although it is evident that the Baltic seals exhibit a compromised endocrine system, which has been associated with high PCB and DDE/DDT levels, further research is needed to elucidate the mechanism of action leading to impaired reproductive performance.

4.1.2.3 Reproductive dysfunction in feral rodents. Adult male white-footed mice (*Peromyscus leucopus*) inhabiting a PCB- and cadmium-contaminated area had significantly lower relative testis weights compared with mice collected at a nonpolluted site (Batty et al., 1990). Seminal vesicle weights in animals from both areas were not significantly different, but there was a higher variability in animals from the contaminated site. The responses of contaminant-exposed individuals were reflected at the population level because the proportion of juveniles and subadults in the summer population did not increase in the PCB- and cadmium-polluted area compared with an increase in the unexposed reference population (Batty et al., 1990). Further research is needed to determine whether these effects can be attributed to PCBs and/or cadmium and if the mechanisms contributing to the observed reproductive effects involve endocrine disruption.

A number of other studies have indicated a contaminant-associated effect on reproduction in wild populations of small rodents. White-footed mice inhabiting a low-PCB-contaminated woodland exhibited a higher population density but greater temporal variability between years and a higher number of transient mice compared with mice from an uncontaminated area (Linzey and Grant, 1994). Meadow voles (*Microtus pennsylvanicus*) inhabiting a chemical waste site at Niagara Falls showed significantly reduced population density and mean life expectancy, as well as liver, adrenal, and seminal vesicle weight, compared with animals from a reference site. Tissues of voles from the waste site contained hexachlorocyclohexane and other chlorinated hydrocarbons. These were not found in tissues of voles from the reference site (Rowley et al., 1983). In a large-scale semi-field study, Pomeroy and Barrett (1975) found that application of a carbamate insecticide Sevin contributed to delayed reproduction and reduced recruitment in cotton rats (*Sigmodon hispidus*). Although these studies indicate that small rodent populations may experience adverse reproductive effects as a result of exposure to environmental chemicals, more research is required to define the mechanism(s) of toxicity.

4.1.2.4 Less-developed case studies. In addition to the species discussed so far, there are numerous others that seem to show signs of endocrine disruption or other adverse physiological effects as a result of exposure to substances with endocrine-disrupting properties. In these cases, however, only inconclusive or circumstantial data are available, which obscures potential links between EDC exposure and fitness in these animals. The following brief examples highlight the wide range of potential effects, which encompass both reproductive and nonreproductive dysfunction (i.e., pathological disorders and altered immunity).

4.1.2.4.1 Reproductive effects. A notable example includes the remaining endangered Florida panthers (*Felix concolor coryi*), which exhibit a variety of defects of the reproductive, endocrine, and immune systems (Facemire et al., 1995). These include a high prevalence of sperm abnormalities, low sperm density, thyroid dysfunction, sterility, and cryptochordism (90% of the male population).

In Alberta, Canada, there are incidences of masculinization (i.e., pseudohermaphroditism) of black and brown bears, but the cause remains unknown (Cattet, 1988). Pseudohermaphroditism was observed in both young (6 months) and old (14 years) female bears. Given the frequency of occurrence, it has been suggested that this effect may be exogenously induced (Cattet, 1988). The herbivorous habits of black and brown bears may expose them to teratogenic herbicides or plant-derived alkaloids that are androgenic. It is also possible that the pseudohermaphroditism is the result of endogenous factors (i.e., excessive maternal androgens) (Benirschke, 1981). Similarly, female polar bears (4 of 269) from Svalbard, Spitsbergen, were described as pseudohermaphrodites by Wiig et al. (1998). This distinction was based on the presence of a 20-mm penis containing a baculum in two of the bears, whereas the other two bears exhibited aberrant genital morphology and a high degree of clitoral hypertrophy. It is also known that PCB levels are high in polar bears from Svalbard (Bernhoft et al., 1997), but no link has been made between the contaminant and endocrine function.

There are numerous other examples suggesting that exposure to OC insecticides and PCBs have affected endocrine function and reproduction in marine mammals. For example, transformation of epididymal and testicular tissue has been observed in North Pacific minke whales (*Balaenoptera acutorostrata*) (Fujise et al., 1998). Hermaphroditism, in a limited numbers of cases, has been observed in beluga whales (*Delphinapterus leucas*) in the St. Lawrence River (De Guise et al., 1994). This has been attributed to PCB/DDT-related hormonal disturbances during stages of early pregnancy,

whereby normal differentiation of male and female organs was disrupted. Subramanian et al. (1987) reported an inverse relationship between plasma testosterone levels and DDE (but not PCB) concentrations in the blubber of Dall's porpoises from the Northwestern Pacific Ocean.

4.1.2.4.2 Nonreproductive effects. Pathological lesions have been reported in several wildlife species that have been exposed to contaminants with known endocrine-disruptive properties. For example, severe adrenocortical hyperplasia, osteoporosis, intestinal ulcers, claw malformations, arteriosclerosis, uterine cell tumors, and decreased epidermal thickness have been reported in Baltic ringed and gray seals (Bergman and Olsson, 1985; Bergman, 1999a, 1999b). These effects have been attributed to PCBs and DDT and their metabolites, notably PCB- and DDE-methyl sulfones and DDD (Lund, 1994), which affect the function of the hypothalamic-pituitary-gonadal and adrenal axes. Pathological disorders, such as adrenal hyperplasia and high incidence of neoplasia, have been reported in beluga whales (*Delphinapterus leucas*) in the St. Lawrence River (De Guise et al., 1994; Martineau et al., 1994). Although these effects were associated with exposure to polyhalogenated aromatic hydrocarbons and PCBs, the involvement of these contaminants in the etiology of the disorders is uncertain.

There is considerable circumstantial and experimental evidence concerning the impact of contaminants with endocrine-disrupting properties on immune function in mammalian wildlife. A confounding factor in discerning whether or not contaminant-induced immune dysfunction results from endocrine disruption is the intricately connected nature of these two systems. Consequently, there is little concrete evidence to support this hypothesis (Reijnders, 1999). One example where this mechanism might be involved is the contaminant-induced immune suppression that has been proposed to contribute to the mass mortalities of marine mammals, including the harbor seal, Baikal seal (*Phoca sibirica*), striped dolphin (*Stenella coeruleoalba*), and bottlenose dolphin (*Tursiops truncata*) (Dietz et al., 1989). In a controlled experiment, female harbor seals fed fish from the polluted Wadden Sea had impaired natural killer cell activity and T-lymphocyte function (de Swart et al., 1994), as well as delayed-type hypersensitivity (Ross et al., 1995) compared with seals fed less contaminated fish from the Atlantic. Although this study showed that contaminants in the fish were immunotoxic, the significance of this finding in terms of impact on survival in the wild is unknown

4.1.3 Conclusion

The current state of the science provides sufficient evidence that feral mammals have been adversely affected by environmental contaminants, but limited evidence exists to support the contention that these effects are mediated through endocrine-dependent mechanisms. Several factors make it difficult to assess the mode(s) of action of environmental chemicals in feral mammals, including a general lack of knowledge regarding their endocrinology/reproductive biology and how other environmental stressors affect these processes.

4.2 Birds

4.2.1 Unique Aspects

There are several aspects of the biology of birds that may make them uniquely vulnerable to potential EDCs. Birds have high metabolic rates and on a weight-adjusted basis often have a higher metabolism and food consumption than placental mammals of similar size. These factors, together with increased rates of metabolic biotransformation of xenobiotics, may contribute to an increased exposure to environmental contaminants. Migration, courtship, breeding, and parental care giving behaviors require high expenditures of energy and are often accompanied by periods of starvation. Birds respond to these situations by mobilizing stored lipid, thereby raising the potential of increased exposure to lipophilic contaminants that are subsequently released.

Bird species vary in terms of the extent of development of the young at hatching. Birds that are in an advanced state of development are called precocial, whereas those in an early stage of development are termed altricial. Relative to the size of the adult bird, the eggs of altricial species are smaller than those of precocial species and contain less yolk.

Altricial species thermoregulate upon hatching and therefore utilize a higher percentage of ingested energy for growth than do precocial birds. In contrast, the demands on the adults of altricial species may be greater due to the relatively narrower limits for brooding behavior (Kleinow et al., 1999). These two developmental strategies could have profound toxicological and toxicokinetic consequences.

There are aspects of sexual differentiation in birds that may make them uniquely sensitive to the effects of EDCs with estrogenic activity. Exposure of an avian embryo to exogenous estrogens during a critical period in development may result in a more adverse effect than would exposure of a mammalian fetus to estrogens during the same critical period. This is explained by the differential roles of estrogens in birds compared with mammals. In birds, estrogen is the differentiating hormone for both the gonads and behavior but is not involved in the differentiation of the gonads in mammals. E_2, in conjunction with other endocrine and paracrine factors, is implicated in the unilateral development of the left ovary and regression of the right ovary. Also, E_2 influences whether the embryonic tissues that differentiate into oviducts and shell gland persist or regress (NRC, 1999). The dramatic sex-reversing effects of early estrogen treatment on male Japanese quail (*Coturnix coturnix japonica*) behavior only occur if the treatment is given before day 12 of the 18-day incubation period (Adkins, 1979), whereas the E_2-induced masculinization of female zebra finch (*Taeniopygia guttata*) is only produced by treatment after hatching (Adkins-Regan et al., 1994). Japanese quail are a precocial species, whereas zebra finches are typical altricial songbirds. The timing of sexual differentiation of behavior in these two species is consistent with the observation that precocial and altricial birds develop similarly but hatch at different stages of the overall developmental sequence (Adkins-Regan et al., 1994).

Carnivorous and especially piscivorous birds as a result of their feeding behavior are exposed to a number of persistent and bioaccumulative organic compounds, many of which are halogenated (Giesy et al. 1994b). Specifically, it has been the persistent chlorinated hydrocarbons that have accumulated to the greatest concentrations and have been related to the most severe adverse effects on the reproductive potential of birds (Kubiak et al. 1989), such as deformities and mortality of embryos (Gilbertson, 1983; Giesy et al., 1994a, 1994b). These effects have contributed to population declines (Peakall, 1986, 1988).

4.2.2 Effect-Based Responses and Case Studies

4.2.2.1 Alterations in behavior. There is evidence from both field and laboratory studies that environmental contaminants from the Great Lakes region influence behavior and reproductive success of

colonial waterbirds. Behavioral abnormalities observed in herring gulls (*Larus argentatus*) from Lake Ontario include aberrant parental behavior involving a failure to sit on eggs or defend nests (Fox et al., 1978). It was suggested that the high levels of chemical contaminants in these birds were the causative factor for the abnormal behavior.

OCs have altered avian reproductive behavior in controlled studies. Ring doves (*Streptopelia risoria*) that were fed a mixture of DDE, PCBs, mirex, and photomirex (contaminants that are found in salmon and gulls of Lake Ontario) exhibited alterations in hormone levels and reproductive behaviors as adults (McArthur et al., 1983). Consumption of the contaminated diets led to a reduction and/or delay in behaviorally induced increases of sex hormones and contaminated females that failed to respond to male courtship behaviors in the normal fashion; pairs receiving the highest dosage spent less time feeding their young. There was a marked dose-related decrease in fledging success, and the breeding cycle was asynchronous in OC-treated birds. The administration of a PCB mixture to adult breeder doves resulted in aberrant incubation (Peakall and Peakall, 1973) and courtship (Tori and Peterle, 1983) behaviors. The PCB-dosed females were particularly affected in the latter experiment and performed only a small number of courtship behaviors, which resulted in a severe impairment of reproductive success. Exposure to the organophosphorus insecticide parathion may also impact avian incubation behavior and reproductive success (Bennett et al., 1991).

Studies with Japanese quail and zebra finches provide substantial evidence that sexual behavioral differentiation in birds is sensitive to androgens and estrogens and that hormonal disturbances can have a profound and permanent change in the reproductive behavior of both sexes (Adkins, 1979; Simpson and Vicario, 1991; Adkins-Regan et al., 1994; NRC, 1999). Although this forms the underlying basis whereby environmental contaminants may modulate behavior, the causative agents and underlying mechanisms responsible for changes in reproductive behavior in wild bird populations are unknown.

4.2.2.2 Abnormal reproductive morphology. Gonadal development may be affected in wild bird populations as a result of exposure to high levels of OCs. Fifty-seven percent of male gull *(Larus argentatus*) embryos collected from Scotch Bonnet Island, Canada, in 1975 and 1976 had testicular feminization (Fox, 1992). Eggs from this site were contaminated with dioxins, PCBs, and mirex (Gilman et al., 1979; Fox, 1992). Similarly, a tern (*Sterna forsteri*) colony showed a high incidence of abnormal testes (Nisbet et al., 1996), but the contaminants responsible for these effects have not been identified. There is also uncertainty as to the interpretation of changes in gonadal morphology in birds because this may represent a normal condition that disappears with age. Additionally, there is little evidence that this widespread feminization is associated with population level effects.

Ovotestis formation in male embryos and retention of the right oviduct in female embryos was observed in experimental studies of gull eggs injected with E_2, diethylstilbestrol and environmental contaminants such as methoxychlor and DDT (NRC, 1999). Altered gonadal development was observed following the injection of *o,p′*-DDT or methoxychlor into western (*Larus occidentalis*) and California (*Larus californicus*) gull eggs on day 1 of incubation (Fry and Toone, 1981; Fry et al., 1987). The injection of methoxychlor and *o,p′*-DDT resulted in the feminization of gonads of male embryos and the persistence of right oviducts in surviving female embryos. These effects were intermediate between embryos from control eggs and eggs injected with E_2. It is difficult to judge the functional significance of these results. The criteria for feminization were a small histological change (i.e., a localization of primordial germ cells in a thickened cortex on the surface of the left testis), and it is not clear whether this change would impact the reproductive success of adult birds. Although these studies point to the possible involvement of the endocrine system, conclusive evidence is lacking.

An attempt to correlate gonadal feminization with OC contamination in a glaucous gull (*Larus hyperboreus*) population in Puget Sound was inconclusive (Fry et al., 1987). A total of 31 adult females from several colonies that span a range of contamination levels were trapped on their nests and sacrificed for gonadal inspection. Interestingly, the lengths of right oviducts were correlated with predicted degrees of chemical contamination. However, the significance of these data is unclear, because all birds were successfully incubating clutches. Furthermore, the rating of the most severe category (>10 mm) is actually similar to the size of a normally occurring vestigial right oviduct (9–10 mm) in the herring gull (Boss and Witschi, 1947), calling into question the relevance of this end point.

4.2.2.3 Sex ratio skew and female–female pairings in gull populations. There is evidence that the sex ratio has been affected in several North American gull populations, resulting in an overabundance of females in some breeding colonies. Associated with changes in sex ratio, there has been an increase in the incidence of female–female pairings in regions contaminated by DDT (Fry et al., 1987; Fox, 1992). A dramatic example occurred in the western gull population on Santa Barbara Island, California, from 1972 to 1978 (Hunt et al., 1980). The incidence of female–female pairings in a colony is usually estimated by documenting the number of nests that contain an abnormally large number of eggs, or "supernormal clutches." Some supernormal clutches arise from female–female pairs, whereas others are associated with polygynous trios of two females and one male (Conover, 1984a). Nests with five or more eggs are usually the result of multiple-female associations (Conover et al., 1979), because a single female gull typically lays one to three eggs. An increase in supernormal clutch incidence was observed in herring gulls inhabiting northeastern Lake Michigan during the period of 1978–1981 (Shugart, 1980; Fitch and Shugart, 1983). Both the California and Great Lakes populations of gulls were exposed to relatively great concentrations of OCs, including DDT, from the 1950s until the 1970s (Fry and Toone, 1981; Fry et al., 1987; Fox, 1992).

Several historical studies have investigated the occurrence of supernormal clutches in the Laridae family utilizing literature sources and museum specimens, in order to determine whether incidences have actually changed in the pre- and post-DDT era. It was discovered that the incidence of supernormal clutches has actually decreased significantly for many species of terns throughout the United States (Conover, 1984b). Supernormal clutch incidence had increased significantly in only three Laridae species since 1950: western gulls and herring gulls nesting in the Great Lakes, and Caspian terns (*Hydroprogne caspia*) breeding in the United States. Supernormal clutches were a regular occurrence in ring-billed (*Larus delawarensis*) and California gulls prior to the DDT era, and their occurrence has not changed over time (Conover and Hunt, 1984a). In contrast, supernormal clutches were not found regularly in western or herring gulls until after 1950, and the sex ratio for their populations as a whole has changed dramatically for both species toward an excess of females. These results supported the hypothesis that the shortage of males at the breeding colonies resulted from a

low male/female ratio in the adult population and not from a failure of feminized males to breed.

A decrease in the ratio of males to females in western and herring gulls could be due to a differential mortality between males and females for each of the two species. Such a differential mortality has not been well studied. It is possible that male gulls could be more susceptible to poisoning from persistent OC contaminants. Male western gulls weigh about 25% more than females, on average, and they feed higher up on the food chain (Pierotti, 1981). Also, male gulls do not have the ability to excrete lipophilic contaminants by laying eggs. For these reasons, it is expected that male gulls might accumulate greater body burdens of these toxicants compared with females. Another possible explanation for the skewed sex ratios observed in gulls from California and the Great Lakes is that exposure to environmental estrogenic contaminants caused either differential male mortality or a feminization of male embryos, which resulted in chemical sterilization and a failed recruitment into the breeding population (Fry et al., 1987). Although this is a plausible hypothesis, there is no direct evidence to support it or the mechanism(s) that involves endocrine disruption.

4.2.2.4 **DDE-induced eggshell thinning.** Toxicant-induced eggshell thinning, primarily caused by DDE (degradation product of DDT), can result in cracked or broken eggs and other adverse reproductive effects (Struger and Weseloh, 1985; Struger et al., 1985; Elliott et al., 1988). Eggshell thinning is mediated by direct effects of DDE on the shell gland (see Chapter 3 for discussion of possible mechanisms), and its occurrence during the period of use of DDT as an insecticide in North America nearly resulted in the extinction of several avian species. Studies in Canadian and Russian peregrine falcon (*Falco peregrinus*) suggest that eggshell thinning continues to be a problem due to the high DDT content in the eggs (Johnstone et al., 1996).

The degree of eggshell thinning varies depending on the sensitivity of the species. For example, eggshell thickness is reduced by more than 30% in response to DDE in brown pelican (*Pelecanus occidentalis*), whereas Japanese quail eggshell thinning ranges from 5% to 15%. In certain species (domestic fowl, *Gallus domesticus*), DDE does not induce eggshell thinning. Many of the species susceptible to eggshell thinning, such as the guillemot (*Uria aalge*) and the double-crested cormorant (*Phalacrocorax auritus*), have experienced dramatic population increases and increased eggshell thickness (see Figure 4.1) since DDT was banned from use (Ludwig, 1984; Price and Weseloh, 1986; Bignert et al., 1994; Weseloh and Ewins, 1994). However, other adverse effects, such as localized impairment of reproductive performance (Tillitt et al., 1992) and anatomical defects (Giesy et al., 1994b) have persisted.

4.2.2.5 **Deformities.** A group of embryonic abnormalities directly related to contaminant exposure in some fish-eating birds has been defined as the specific syndrome GLEMEDS (Gilbertson and Fox, 1977; Gilbertson et al., 1991). GLEMEDS involves a consistent pattern of subcutaneous edema, beak malformations, cardiac edema, and skeletal malformations (Fox et al., 1991; Ludwig et al., 1993; Gilbertson et al., 1991), and particularly abnormalities that are of ectodermal origin (Rogan et al., 1988). An abnormality that has been characterized well in cormorants (crossed-bill syndrome) has been correlated with concentrations of different polychlorinated halogens in bird eggs (Fox et al., 1991). The expression of this syndrome is the result of the deposition of coplanar PCB congeners in the eggs by the maternal bioaccumulation of the compounds that were present in their fish-based diet (Gilbertson et al., 1991).

Declines in concentrations of DDT, PCBs, and PCDDs/PCDFs in the Great Lakes have been associated with increasing populations of herring gulls and double-crested cormorants, as well as other fish-eating birds. There have also been reductions in the rate of reproductive failure and the symptoms of GLEMEDS (Gilbertson et al., 1991; Grasman et al., 1998). Nevertheless, biochemical effects associated with persistent OC exposure persists in all species of fish-eating colonial water birds inhabiting Lake Ontario and the other Great Lakes (Fox et al., 1991; Fox, 1993). Polychlorinated halogens also affect the concentrations of both blood and liver levels of vitamin A. Because vitamin A is necessary for normal embryonic development (Twal and Zile, 1997), it is possible that altered levels of vitamin A contribute to the birth defects in birds.

4.2.3 Conclusion

In birds, the oviparous reproductive strategy and certain life history traits create avenues of exposure that may make these species more vulnerable to EDCs than are traditional animal models or humans. Although exposure to environmental contaminants can have dramatic effects (i.e., eggshell thinning) on endocrine-regulated

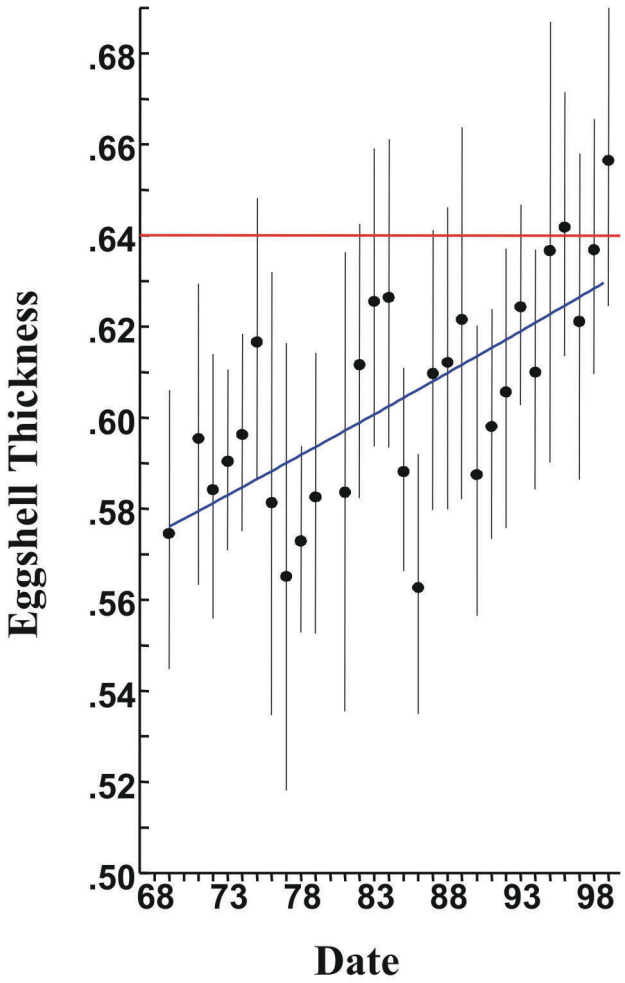


Figure 4.1. Eggshell thickness index [shell weight/(shell length × shell breath × shell thickness)] of guillemot (*Uria aalge*) eggs from the Baltic during 1968–1998 (blue line). The red line indicates the eggshell thickness index of pre-1946 guillemot eggs from the same nestling colony (modified from Bignert et al., 1994).

processes (reproduction) and overall population fitness, the mechanism need not involve endocrine disruption. Likewise, the same individuals may experience endocrine disruption, which may or may not be linked to effects on reproduction and population fitness.

4.3 Reptiles

4.3.1 Unique Aspects

Reptiles encompass a broad diversity of groups, including squamates (lizards and snakes), turtles and tortoises, crocodilians (crocodiles and alligators), and sphenodon (tuatara). Their evolutionary history has given them a suite of unique phylogenetic, anatomical, physiological, and ecological characteristics that must be considered when evaluating the potential effects of environmental EDCs (Lamb et al., 1995; Palmer et al., 1997; Crain and Guillette, 1998). Also, endocrine systems are highly divergent across this class, which confounds efforts to make predictions of the physiological responses across reptilian species.

This phylogenetic background has led to a diversity of reproductive and developmental characteristics. For instance, although all tuatara, turtles, and crocodilians are oviparous, the squamates exhibit both oviparity and viviparity (Palmer et al., 1997). Even among the oviparous species, there is significant variation in the anatomy of the female reproductive tracts (Palmer and Guillette, 1988, 1990, 1992). In all species of reptiles, offspring are formed as miniature copies of the adults, without larval stages. The eggs of most oviparous reptiles are buried, providing a route for embryonic exposure via the substrate. Although an eggshell surrounds the embryo, dissolved compounds readily cross into the egg.

Reptiles exhibit a variety of sex-determining mechanisms, including genetic and environmental sex determination, such as TSD, which occurs among all crocodiles, most turtles, and many lizards (Lance, 1994). In TSD reptiles, the incubation temperature of the eggs determines whether the offspring will be male or female. However, there is tremendous variation in the pattern of TSD among reptiles (Wibbels et al., 1998). This variation complicates the understanding of potential influences that EDCs may have on the process of sex determination in reptiles.

Steroid hormones (estrogens, testosterone) have been shown to override the effects of temperature on sex determination in TSD species (Wibbels and Crews, 1995). For example, the administration of exogenous estrogens results in female sex determination, even though the eggs are incubated at all-male-producing temperatures. Likewise, several environmental chemicals (PCBs, *trans*-nonachlor, *cis*-nonachlor, chlordane, and *p,p′*-DDE) have been shown to alter turtle sex determination (Bergeron et al., 1994; Crews et al., 1995; Willingham et al., 2001). In addition, PCBs and chlordane also alter steroid hormone profiles of hatchling turtles (Willingham et al., 2001). The mechanism by which hormones and putative EDCs influence sexual determination remains unclear. Because testosterone serves as the precursor to both E_2 and 5α-dihydrotestosterone, it has been hypothesized that the TSD phenomenon in reptiles results from competition between 5α-reductase and aromatase (Crews and Bergeron, 1994; Jeyasuria and Place, 1998). Aromatase activity is associated with female-producing temperatures in the turtles *Emys orbicularis* (Desvages and Pieau, 1992), *Dermochelys coriacea* (Desvages et al., 1993), and *Chelydra serpentina* (Rhen and Lang, 1994), and in the slider turtle *Trachemys scripta* (Crews and Bergeron, 1994). Aromatase mRNA is elevated in the putative ovary but not in the putative testis from the diamondback terrapin *Malaclemys terrapin* (Jeyasuria and Place, 1997; Jeyasuria and Place, 1998). In the red-eared slider, aromatase activity was significantly elevated in the brain in females, although there was no difference between sexes for the adrenal-kidney-gonadal axis. Further, treatment with aromatase inhibitors indicates that aromatase is a key enzyme involved in sex determination in many reptiles (Jeyasuria et al., 1994; Richard-Mercier et al., 1995; Rhen and Lang, 1994; Crews and Bergeron, 1994). E_2 modulates the regulation of steroidogenic factor 1 during gonadogenesis in turtles (Fleming and Crews, 2001).

Other environmental factors besides temperature play a role in the sexual determination of reptiles, and these may further obscure cause-and-effect relationships between potential EDCs and alterations of endocrine function. These factors include alterations in the hydric environment (Gutzke and Paukstis, 1983), CO_2 levels (Jeyasuria and Place, 1998), and higher temperatures that lead to a decrease in pH within the egg. Because 5α-reductase has a strong pH dependence curve, temperature may act indirectly on sexual differentiation by altering pH levels, which subsequently affects the activities of key steroid metabolizing enzymes (Etchberger et al., 1992).

The feeding strategies of reptiles may increase their capacity to bioaccumulate environmental toxins. Reptilian feeding ecology ranges from herbivory to carnivory, with some of the carnivorous reptiles being at or near the top of the food web. Many reptiles, such as turtles, crocodilians, and large snakes, are long-lived, with life expectancies in the wild that surpass 30 years (Bowler, 1977; Gibbons and Semlitsch, 1982; Congdon et al., 1983). This provides time for significant bioaccumulation of environmental contaminants in their tissues. In fact, reptiles have been shown to bioaccumulate and biomagnify contaminants to levels equal to or greater than birds and mammals (Olafsson et al., 1983; Hall and Henry, 1992; Cobb and Wood, 1997).

4.3.2 Effect-Based Responses and Case Studies

4.3.2.1 Developmental abnormalities in Lake Apopka alligators. Alligators (*Alligator mississippiensis*) in Lake Apopka, Florida, USA, provide one of the most publicized examples of EDC effects on a wildlife population. In 1980, a stream that feeds Lake Apopka was contaminated with high concentrations of dicofol (including its metabolites DDD, DDE, and chloro-DDT) and other compounds after a chemical spill. Shortly thereafter (1980–1984), the population of alligators declined by 90% (Guillette et al., 1994). A decline in clutch viability was not seen until 1984. Lake Apopka alligators have elevated concentrations of *p,p′*-DDE, dieldrin, endrin, mirex, oxychlordane, DDT, and PCBs (Guillette et al., 1999a). This depressed clutch viability continues today, leading to a depressed population of adult alligators.

Juvenile alligators from Lake Apopka showed a variety of developmental abnormalities (abnormal gonadal morphology, altered gonadal steroidogenesis, and changes in sex steroid concentrations in males and females), which have been attributed to contaminants that disrupt endocrine function. The evaluations of these end points were made in comparison with alligators at a relatively uncontaminated site, Lake Woodruff, Florida. Specifically, the male and female juvenile alligators from Lake Apopka had depressed plasma testosterone levels and elevated E_2 levels, respectively (Guillette et al., 1994, 1999a). Also, there were alterations in the steroid (i.e., E_2, testosterone) biosynthetic capacity of ovarian and testicular tissue *in vitro* (Guillette et al., 1995; Crain et al., 1997). Typically, hormone concentrations in developing organisms are related to the developmental stage or the size of the organism. As expected, steroid and thyroid hormone

levels were related to body size in juvenile alligators from Lake Woodruff but not in juvenile alligators from Lake Apopka (Crain et al., 1998a, 1998b).

Alligators at Lake Apopka showed a suite of gonadal deformities that correlated with abnormal plasma steroid levels (Guillette et al., 1994, 1996). In females, some follicles were found to be polyovular (consisting of three or four oocytes), and selected oocytes were polynuclear (possessing two or three nuclei each) (Guillette et al., 1994). The testes of male alligators exhibited poorly organized seminiferous tubules, and many were lined with a cuboidal epithelium. In addition, alterations to sperm cells were identifiable by the presence of elongated, bar-shaped nuclei (Guillette et al., 1994). Male alligators showed a significantly reduced phallus size that, at Lake Apopka, varied between areas with different levels of pollution (Guillette et al., 1996; see Figure 4.2). It is unclear whether the male and female alligators exhibiting these gonadal deformities could be sexually potent. Further research indicates that alterations in steroid hormone concentrations and phallus size are widespread in Florida lakes (Guillette et al., 1999b).

Although the principal contaminant (*p,p′*-DDE) in eggs and juvenile alligators at Lake Apopka has been identified (Vos et al., 2000), the specific chemical(s) responsible for the observed effects is unknown (Ankley and Giesy, 1998). Several hypotheses have been proposed to explain the contaminant-induced endocrine disruption. For example, multiple EDCs may interact with the alligator estrogen receptor, and *p,p′*-DDE could act as an androgen antagonist in embryonic and juvenile alligators (Guillette et al., 1996; Crain and Guillette, 1998). In addition, the contaminant-induced mechanism of endocrine disruption may involve alterations in aromatase enzyme activity and disruptions in the thyroid-gonad axis (Crain et al., 1997, 1998).

4.3.2.2 **Developmental abnormalities in Great Lakes snapping turtles.** The developmental abnormalities observed in common snapping turtles (*Chelydra serpentinia serpentinia*) in the Great Lakes–St. Lawrence River basin (Bishop et al., 1998) offer another possible example of the effects of EDCs in reptiles. A report by Bishop et al. (1991) showed that unhatched eggs and hatchling deformities in the snapping turtles occurred from sites with the highest concentrations of chlorinated hydrocarbons during 1986–1988. Bishop et al. (1998) determined that, of the numerous chemicals identified, concentrations of PCBs, PCDDs, and PCDFs in eggs during 1989–1991 were significantly related to the occurrence of abnormalities in developing turtles. Several hatchling abnormalities were identified, including absent or altered tails, carapace anomalies (missing or extra scutes), unresorbed yolk sacs, and fore and hind limb deformities. Also, it has been suggested that EDCs may affect sexually dimorphic morphology of adult snapping turtles. In snapping turtles, the ratio of the PPR is sexually dimorphic. A report by de Solla et al. (1998) found that adult turtles (*Chelydra serpentina serpentina*) from sites contaminated with OCs exhibited significant reductions in PPR compared with reference site turtles. The same turtles did not show alterations in the levels of sex steroids (i.e., E_2 and testosterone). Controlled studies of the effects of polychlorinated hydrocarbons on the endocrine physiology (and reproductive fitness) of turtles are needed to establish cause-and-effect relationships.

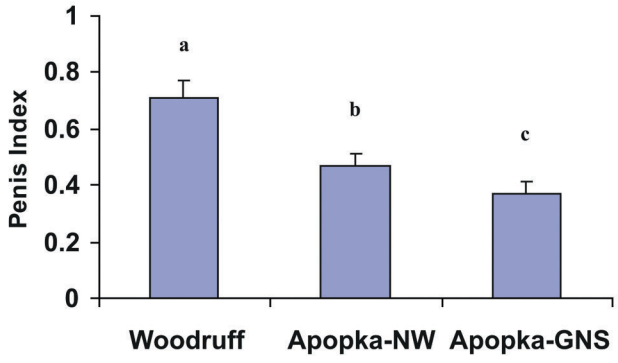


Figure 4.2. Mean phallus size in alligators (*Alligator mississippiensis*) from two regions from a control lake (Lake Woodruff; $n = 40$) and two regions of a contaminated lake (Lake Apopka). Lake Apopka samples are separated into two localities, the Gourd Neck Spring area (Apopka-GNS; $n = 34$), where contaminants from the Tower Chemical spill entered the lake, and the northwestern part of the lake (Apopka-NW, $n = 20$), which is farther from the spill. Penis size is represented as an index [(penis tip length × penis base width)/snout vent length]. The letters a, b, c indicate significant differences ($p > .05$) among Lake regions. Redrawn from Guillette et al. (1996).

4.3.3 Conclusion

Reptiles have received little attention from an ecotoxicological perspective. It is clear that some developmental processes in reptiles, particularly sex determination, gonadal development, steroid hormone synthesis, and development of secondary sex characteristics, are susceptible to endocrine disruption. Although some reptile populations have been impacted by environmental contaminants with endocrine-disrupting properties, it is unclear how widespread the phenomenon is. Currently, there is insufficient data to evaluate whether aquatic reptiles are at greater risk of endocrine disruption compared with terrestrial reptile species, for which we have limited data.

4.4 Amphibians

4.4.1 Unique Aspects

Metamorphosis occurs almost universally in Amphibia and is exhibited in the vertebrate groups Agnatha (jawless fishes) and Osteichthyes (bony fishes) as well. The remaining vertebrates [Chondrichthyes (cartilaginous fishes), reptiles, birds, and mammals] do not exhibit metamorphosis (Norris, 1983). The life histories of extant amphibians are diverse, with some species experiencing complex transformations that progress from free-swimming aqueous life stages to terrestrialness. These transformations involve a myriad of structural and biochemical changes to processes such as respiration, osmoregulation, waste (nitrogen) excretion, and locomotion. Therefore, this class of vertebrates may be subject to EDC exposures during different stages of their life cycle and may be particularly at risk of the effects of EDCs (Vos et al., 2000). The endocrine regulation of metamorphosis is composed of several developmental hormones that could be affected by EDCs, particularly the thyroid hormones (triiodothyronine/thyroxine), but also corticosteroids, prolactin, and RA derivatives (Norris, 1996). It has been postulated that water-soluble and nonpersistent chemicals may affect metamorphosis. It is unlikely that these putative EDCs would be identified in traditional ecotoxicological assays, which concentrate on persistent, bioaccumulative chemicals. Currently, there is insufficient data to discern whether or not metamorphosis in amphibians is especially sensitive to the effects of aquatic contaminants with endocrine-disrupting properties.

Amphibians may be exposed to EDCs via several different routes because of their semipermeable skin, the development of their

eggs and gill-breathing larvae in the water, and their position in the food web, which changes from herbivory of tadpoles to carnivorous adults (Gutleb et al., 1999). Hibernation is another life history trait of many amphibians, during which time species remain submerged in the substrate, which may make them more vulnerable to toxins. It has been suggested that amphibians could be more susceptible to the effects of environmental contaminants during hibernation (Russell et al., 1995), because the same doses of DDT have been found to be only moderately toxic to adult common frogs (*Rana temporaria* L.) in oral doses but results in mortality under conditions of food deprivation (Harri et al., 1979).

4.4.2 Effect-Based Responses and Case Studies

4.4.2.1 Amphibian population changes. Currently, there is little dispute within the scientific community that adverse population-level effects in amphibians are occurring (Pechmann et al., 1991; Pechmann and Wilbur, 1994). Populations are declining in both pristine and polluted habitats worldwide (Vos et al., 2000), and there have been several recent reviews that describe the amphibian populations that are affected (Sarkar, 1996; Green, 1997a, 1997b; Lannoo, 1998; Corn, 1999). A variety of changes in amphibian populations have been noted, including species extinctions, decreases in population size, and changes in the spatial distribution of certain species. Primarily, efforts have concentrated on alterations of amphibian populations in North and Central America, as well as in Australia and Europe (Corn, 1999). Comparatively little is known concerning the status of amphibian populations in Asia and Africa.

A study by Carey and Bryant (1995) examined the potential for environmental contaminants to affect amphibian population numbers through the disruption of growth and development of the young. Their conclusion was that critical data are lacking in most documented cases, which precluded drawing a link between altered populations and chemical exposures. Currently, there is little evidence to support the hypothesis that the effects observed in amphibians in the field are the result of EDCs. An exception is the report by Kirk (1988), which showed a strong association between DDT spraying and effects on a local population of western spotted frogs (*Rana pretiosa*). Several other reports are more speculative (Drost and Fellers, 1996; McConnell et al., 1998). For example, in 1993, Russell et al. (1995) examined a population of spring peepers (*Pseudacris crucifer*) at Point Pelee National Park, Canada, and found that these frogs had appreciable levels of DDT, DDE, DDD, and dieldrin. This locality was the site of frequent DDT spraying until 1967. Since 1972, cricket (*Acris crepitans*), gray tree (*Hyla versicolor*), and bull (*R. catesbeiana*) frogs have become locally extinct. Researchers have not been able to attribute the declines in amphibian populations to obvious environmental factors (i.e., habitats have actually improved and acid rain has been ruled out). The authors suggested that the application of pesticides decades ago might have been an important factor in the decline of amphibians at this park. In these examples, the mechanism of action of the putative agents is uncertain, and it is unknown whether the mechanism was endocrine mediated.

There is limited evidence from controlled laboratory studies that amphibians are affected by known EDCs in a manner consistent with other vertebrate species (Hall and Henry, 1992). For example, vitellogenesis is induced in African clawed frogs (*Xenopus laevis*) by exposure to *o,p*-DDT, dieldrin, and toxaphene (Palmer and Palmer, 1995; Palmer et al., 1997). As discussed by Carey and Bryant (1995), EDCs may affect amphibians either directly (alterations in developmental hormones) or indirectly (increased disease susceptibility associated with compromised immunity).

4.4.2.2 Deformities in amphibians. There have been several recent reports of deformed frogs in abnormally large numbers throughout North America (Ouellet et al., 1997; Schmidt, 1997; Ankley and Giesy, 1998). Most commonly, the affected species appear to be ranids, such as the northern leopard frog (*Rana pipiens*), green frogs (*R. clamitans*), and the mink frog (*R. septentironalis)*. The predominant malformations include missing or supernumerary limbs, bony limblike projections, digit and musculature malformities, and eye and central nervous system abnormalities (Ankley and Giesy, 1998b). It is unknown whether the instances of population declines can be attributed to the deformities, and/or whether the same environmental factor(s) is responsible for both of these effects.

Despite the absence of a cause-and-effect relationship between EDCs and amphibian deformities, there is a strong mechanistic basis for a chemical-based etiology of amphibian malformations. It has been postulated that environmental retinoids, or "retinoid mimics," may contribute to the induction of developmental effects of ranids in the wild (Ankley and Giesy, 1998b). In addition to thyroid hormones, early embryonic development in amphibians is strongly dependent on the RA hormonal system composed of active retinoids (i.e., all-*trans*-RA, 9-*cis*-RA, and structurally related derivatives) and a number of retinoid receptors (Schena, 1989). The RA system controls different developmental processes including pattern formation and limb development (Wagner et al., 1990; Shimeld, 1996). RA treatment during embryogenesis can result in developmental abnormalities as was shown in case of *Xenopus laevis* embryos (Papalopulu et al., 1991), and limb defects have been reported in a variety of other vertebrate models (Maden, 1993; Rutledge et al., 1994; Scott et al., 1994). A recent study by Gutleb et al. (1999) reported that a non-*ortho* PCB congener (PCB 126) caused dose-related malformations (edema, lack of gut coiling, misformed eyes and tails) in *X. laevis* embryos and that retinoid concentrations were significantly altered in PCB-dosed embryos. A metabolite of the environmental contaminant methoprene has been shown in transfected cell lines to activate an RA receptor (Harmon et al., 1995), which has prompted speculation that this pesticide could be involved in the production of limb malformations in native anurans. However, studies are required to validate this hypothesis. Further research is needed to elucidate whether or not environmental contaminants are responsible for the observed malformations in amphibians, and if so, whether or not these effects are mediated through endocrine-disrupting mechanisms of action.

Other factors besides chemical stressors need to be considered as possible causative agents of amphibian malformations. Amphibian limb malformations may be due to the physical disruption of limb buds by trematode cysts (Sessions and Ruth, 1990). Certain trematodes, which utilize larval amphibians as a secondary host, can occur at relatively large concentrations as metacercaria in the developing limb bud field. A recent study showed that severe limb abnormalities were induced in Pacific tree frogs (*Hyla regilla*) exposed to cercariae of a trematode parasite (Johnson et al., 1999). These abnormalities closely matched those observed at field sites. In addition, it has been postulated that increased ultraviolet exposure, associated with stratospheric ozone depletion, may be responsible for some amphibian malformations (UNEP, 1998). Controlled experiments have shown that ultraviolet light, as well as natural sunlight, can cause hind limb malformations (ectromelia and ectrodactyly) in the northern leopard frog (Ankley et al., 1998b).

4.4.3 Conclusion

Currently, there are insufficient data to implicate EDCs as causative agents in amphibian declines. Also, there is not enough conclusive evidence to state that environmental contaminants are responsible for the observed malformations. Clearly, further work is needed to assess this possibility. It is worthy to note, however, that increasingly more studies are focusing on amphibians as potential target species of EDCs (Harris et al., 1998a, 1998b).

4.5 Fish

4.5.1 Unique Aspects

Fish are the most successful group of vertebrates and display a high degree of heterogeneity in their physiology, anatomy, behavior, and ecology. There are over 3,000 species of cartilaginous fish (elasmobranchs and chimaeras), over 20,000 species of bony fish (teleosts, dipnoans, and holosteans), and fewer species of the ancient jawless fish (lampreys and hagfish). Only a small proportion of fish species has been thoroughly investigated, and data on cyprinids and salmonids (both teleosts) dominate the literature.

Fish have evolved to inhabit a range of aquatic environments (freshwater, brackish, and marine) that differ in osmotic properties. Both aquatic respiration and osmoregulation may contribute to an increased exposure of EDCs to fish. The higher ventilation rate of fish compared with humans may increase their exposure to waterborne contaminants to the respiratory surfaces of the gills (Van Der Kraak et al., 2001). In addition, other features of fish gills (countercurrent system of blood and water flow, thin epithelial membranes, and a high surface area) may increase the uptake of compounds from the water and their transfer to the bloodstream. Marine teleosts drink seawater due to their hypotonic nature, which may contribute to their exposure to waterborne substances. In contrast, hyposmotic freshwater fish (these fish do not drink) move water into their bodies, thereby creating a route of exposure to waterborne contaminants.

A high degree of diversity is displayed in the reproductive strategies of fish (Kime, 1998). The majority of fish are oviparous, but species displaying ovoviviparity and true viviparity are also widespread. Of the oviparous species, reproductive strategies range from the production of many small eggs (up to 28 million in a single cod) that are released into the plankton, to fewer and larger demersal eggs, which may be laid in a nest and guarded. In addition, some elasmobranchs have very few large, yolky eggs. A significant route of exposure for early-life-stage fish is through the maternal transfer of hydrophobic xenobiotic contaminants sequestered in lipid reserves. This route of exposure may be greater than the levels accumulated directly from the water column. Also, the limited activity of biotransformation and excretory enzymes of early life stages may maximize exposure during critical periods of development (Van Der Kraak et al., 2001).

Sexual plasticity is another reproductive strategy in certain species of fish. Although fish are usually gonochoristic (separate sexes), functional hermaphrodites do exist (Serranidae and Sparidae), which is uncommon among vertebrates (Chan and Yeung, 1983). In some species of teleost, particularly coral reef fishes, individuals can reversibly change from one functional sex to the other in response to environmental (temperature) and social cues (pheromones) (Stahlschmidt-Allner and Reinboth, 1991). It is unknown how the sexual plasticity that occurs in some species of fish may be affected by environmental contaminants with endocrine-disrupting properties. However, the existence of this natural sex change implies that irreversible sexual imprinting does not occur in fish in the same manner as it does in the brain of fetal mammals.

4.5.2 Effect-Based Responses and Case Studies

4.5.2.1 Induction of vitellogenesis in juvenile or male fish. The induction of VTG production in juvenile or male fish in the field has become one of the most notable and convincing biological responses of fish linked to EDC (i.e., estrogenic compounds) exposure (Tyler and Routledge, 1998; Kime et al., 1999). Currently, there are numerous cases of VTG induction in fish in a variety of water bodies across Europe, Japan, and North America. The most dramatic increases in VTG levels appear to be in fish exposed to STW discharges at sites in the United Kingdom. The implications of these responses in both freshwater and marine fish in terms of reproductive success and viability are unknown.

Research by Purdom et al. (1994) found that male rainbow trout (*Oncorhynchus mykiss*) caged in STW discharges in England had elevated plasma VTG levels (up to 147 mg/ml) that were equal to or exceeded the levels found in mature females. Caged immature carp (*Cyprinus carpio*) also showed elevated VTG levels, but to a lesser extent. Subsequent work by Harries et al. (1996, 1997) showed that several rivers in the United Kingdom also contained sufficiently high concentrations of estrogenic compounds to induce vitellogenesis in male rainbow trout. In addition to VTG induction, fish exposed downstream of discharges sometimes exhibited enlarged livers and reduced testicular growth. Typically, the degree of vitellogenesis declined rapidly with distance from the source of pollution, and much of this was attributed to dilution of the active compounds (Harries et al., 1999). Also, induction of plasma VTG in chub (*Leuciscus cephalus*) has been observed in the River Moselle in France downstream of urban areas (Flammarion et al., 2000).

There are several more examples of VTG induction in freshwater fish exposed to municipal sewage discharges. Different localities in the USA revealed similar responses in male carp (Bevans et al., 1996; Folmar et al., 1996; Goodbred et al., 1997) and fathead minnows (*Pimephales promelas*; Nichols et al. 1999) exposed to municipal wastewaters. In Sweden, the bile of juvenile rainbow trout caged in STW discharge for 2 weeks contained the natural estrogens E_2 and estrone and the synthetic estrogen 17α-ethinyl estradiol, and plasma VTG levels were high (1.5 mg/ml) (Larsson et al., 1999).

VTG inductions in the documented cases discussed above have been attributed primarily to environmental estrogens that are either synthetic or natural compounds. For example, in the River Aire, NP and its ethoxylates, which were previously discharged into the water in large amounts, have been implicated as causative agents in the VTG responses of the fish. In controlled laboratory studies with rainbow trout, Jobling et al. (1996) demonstrated dhat NP, at environmentally relevant concentrations (as low as 20 µg/liter), was able to induce VTG production and retard testicular growth. The natural (i.e., E_2, estrone) and synthetic (i.e., ethynyl estradiol) estrogen hormones may contribute substantially to the observed responses (Routledge et al., 1998). These hormones enter the sewage as inactive conjugates but can be deconjugated into active compounds by bacterial enzymic activity (Panter et al., 1999). As a result of measuring a number of estrogenic compounds in fish bile, another estrogenic compound, bisphenol A, has also been added to the list of probable causative agents (Larsson et al., 1999).

The induction of VTG is not confined to only freshwater fishes. VTG induction and testicular abnormalities were detected in male European flounder (*Platichthys flesus*) that were caught near an STW discharge in the Tyne estuary, United Kingdom (Lye et al., 1997,

1998). Later work partly associated these effects with exposure to, and bioaccumulation of, several estrogenic alkylphenols (Lye et al., 1999). Subsequent investigations in Britain (Matthiessen et al., 1998; Allen et al., 1999a, 1999b) have measured high levels of VTG (up to 20 mg/ml plasma) in male flounder from heavily industrialized estuaries (i.e., Tyne, Tees, Wear, and Mersey estuaries; see Figure 4.3). In contrast, low or undetectable levels of VTG were found in male flounder from estuaries draining rural areas or urban areas with relatively little heavy industrial activity. Similar effects to those described in flounder from the United Kingdom have also been observed in Japanese flounder (*Pleuronectes yokohamae*) from Tokyo Bay (Hashimoto et al., 2000). The causative substances have not yet been fully identified, but as with freshwaters, mixtures of natural and synthetic estrogenic substances have been detected in estuarine waters. However, most estuarine estrogenic activity appears to be adsorbed on the sediment solid phase, yet the majority of this activity remains to be identified (Thomas et al., 2001).

Pulp and paper mill effluents may also contain estrogenic compounds that alter VTG production in exposed fish. In one of three mills studied in Finland, VTG gene expression was induced in caged whitefish (*Coregonus lavaretus* L.) held for 4 weeks in the effluent (Mellanen et al., 1999). This mill was found to discharge considerably more wood-derived compounds (sterols, resin acids) than did the other two mills. Pelissero et al. (1991a, 1991b) demonstrated that VTG was produced in male and juvenile sturgeon (*Acipenser baeri*) exposed in the laboratory to a variety of phytoestrogens. In addition, controlled laboratory studies by Tremblay and Van Der Kraak (1998, 1999) demonstrated that exposure of sexually immature rainbow trout either to BKME or to β-sitosterol (a phytosterol found in the effluent) for 3 weeks results in the induction of vitellogenesis. However, field studies of feral white sucker (*Catostomus commersoni*) downstream of BKME discharge in Canadian waters have failed to show VTG induction (Van Der Kraak et al., 1998b).

4.5.2.2 Reproductive abnormalities.

4.5.2.2.1 Altered gonadal development induced by STW discharges. The first documented cases of abnormal gonadal development in fish were those found to occur concomitantly with VTG induction discussed in the previous section. Again, a large proportion of the data has been generated from studies of fish exposed to STW discharges in the United Kingdom. These fish are indisputably affected by contaminants with estrogenic activity. The inhibition of testicular growth has been observed in adult rainbow trout held in waters heavily contaminated with estrogenic compounds such as in the River Aire, which is contaminated with alkylphenols (Harries et al., 1997). Trout exposed in laboratory studies to environmentally realistic concentrations of alkylphenols display similar effects on testicular growth (Jobling et al., 1996). Also, occurrences of ovotestis have been documented in wild male roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*) collected downstream of STW discharges in several rivers in the United Kingdom. The proportion of fish found with ovotestis varies from just a few percent to 100% in the case of wild roach in some rivers (Aire and Nene).

The severity of the ovotestis condition ranges from the occasional oocyte in otherwise normal testicular tissue, to large regions of mature ovarian tissue interspersed with abnormal testicular tissue. Presumably, this condition is caused by exposure of the fish to estrogenic compounds during critical stages of gonadogenesis (Jobling et al., 1998a; Tyler and Routledge, 1998). Other testicular abnormalities documented in some of the fish exposed to STW discharges include feminized or absent vas deferens and impaired milt production (Jobling et al., 1998b). Feminization of the vas deferens has been described in all-male carp larvae exposed

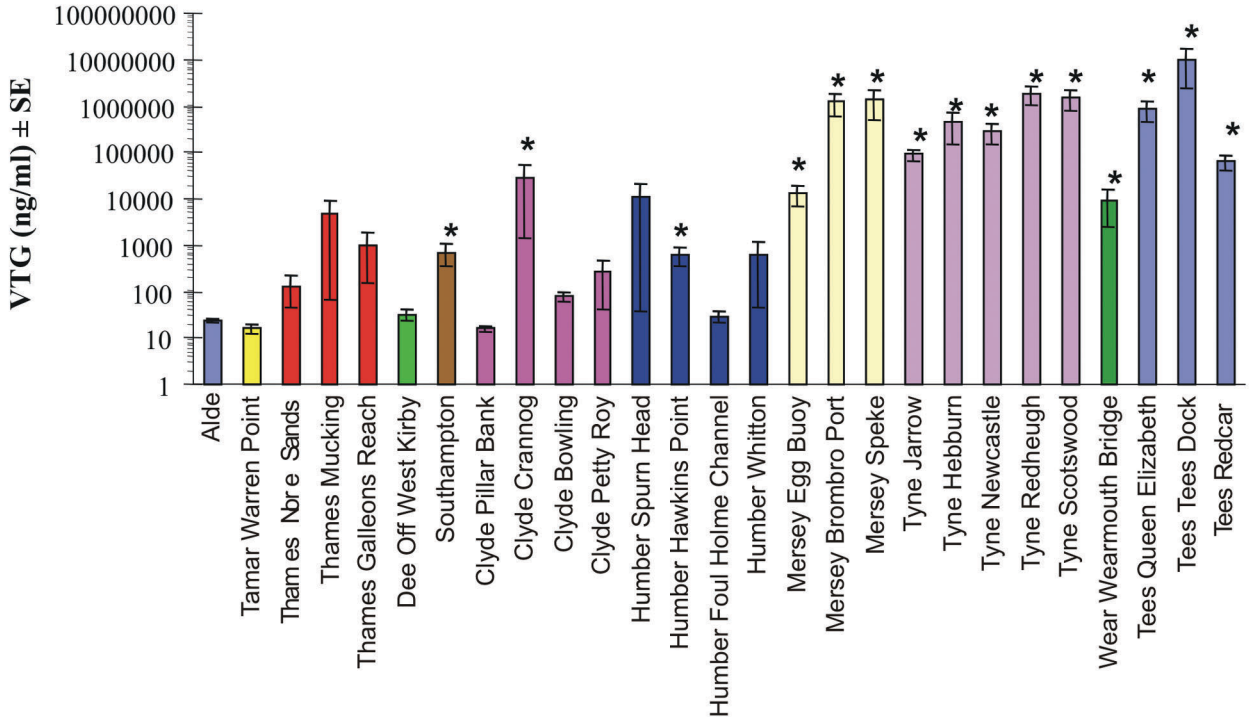


Figure 4.3. Mean concentrations of plasma VTG in male flounder (*Platichthys flesus*) from various U.K. estuaries sampled in 1997. Asterisks indicate significant difference from the clean reference estuary, Alde (extreme left). Reproduced by permission of the United Kingdom Centre for Environment, Fisheries, and Aquatic Science (CEFAS).

in the laboratory to 4-*tert*-pentylphenol (Gimeno, 1997; Gimeno et al., 1996, 1998a, 1998b).

Wild European flounder from heavily industrialized estuaries (the Mersey and Tyne) and a bay (Seine) were reported to have altered spermatogenesis (Lye et al., 1998), and up to 18% showed ovotestis (Matthiessen et al., 1998; Allen et al., 1999a, 1999b; Minier et al., 2000; see Figure 4.4). However, most estuaries do not contain any flounder with ovotestis. It has been hypothesized by Matthiessen et al. (1998) that flounder do not show higher prevalences of ovotestis (in estuaries where VTG is nevertheless induced) because their larvae probably undergo gonadogenesis while still at sea, under relatively uncontaminated conditions. Although the causes of these effects in U.K. estuaries are unknown, circumstantial evidence suggests that endocrine disruption from natural and synthetic estrogenic compounds may be responsible (Lye et al., 1999; Thomas et al., 2001). Ovotestis has also been observed in OC-contaminated shovelnose sturgeon (*Scaphirhynchus platyorynchus*) living in the Mississippi River downstream of St. Louis, Missouri (Harshbarger et al., 2000). Finally, Batty and Lim (1999) have shown that male mosquitofish (*Gambusia affinis*) living downstream of a sewage treatment plant in Australia possess gonopodia of reduced size. The gonopodium is an anal fin modified during sexual development for the deposition of sperm, but it is unknown if reduced size affects fertilization success.

4.5.2.2.2 Reproductive abnormalities induced by pulp and paper mill effluents. Reproductive abnormalities have been reported in fish exposed to pulp and paper mill effluents. Primarily, field studies have focused on the effects of effluents on fish (white sucker, longnose sucker *Catostomus catostomus*, lake whitefish *Coregonus clupeaformis*) in Canadian waters (Munkittrick et al., 1991; Gagnon et al., 1995; Van Der Kraak et al., 1998b; Munkittrick et al., 1998), but similar effects have been reported in fish (Eurasian perch *Perca fluviatilis*, blenny *Zoarces viviparus*) in Scandinavia (Andersson et al., 1988; Neuman and Karås, 1988; Sandström et al., 1988; Larsson et al., 1997).

In Canada, white sucker collected downstream of BKME outfalls at Jackfish Bay, Lake Superior, exhibited a wide array of altered reproductive responses, including reductions in gonad size, delayed sexual maturity, and reduced expression of secondary sexual characteristics (decreased nuptial tubercles on males) compared with control sites (Van Der Kraak et al., 1998b; see Figure 4.5). The viability of eggs and sperm and the viability of developing larvae of these suckers appeared to be normal (McMaster et al., 1992). Similar reproductive effects have been observed in laboratory studies using fathead minnows exposed to BKME (Kovacs et al., 1995). Lake whitefish collected at Jackfish Bay also exhibited reproductive abnormalities and delayed sexual maturation (Munkittrick et al., 1992a, 1992b). In perch, similar effects (reduced gonad size and delayed sexual maturity) have been observed, coupled with impaired fry production, smaller embryos, increased larval mortality, and reduced abundance (Larsson et al., 1997). Effects observed downstream of pulp and paper mill discharges tend to decrease with increasing distance from the outfalls. In most cases, there is sufficient evidence to conclude that the effluents are responsible for the observed effects.

The active compounds responsible for the reproductive abnormalities in BKME-exposed fish have not been identified. However, several compounds may contribute to the effects, including β-sitosterol, other sterols, lignans, stilbenes, and resin acids, which are compounds with weak estrogenic activity (Adlerkreutz, 1988; Mellanen et al., 1996). Other possible compounds are stigmastanol and a β-sitosterol degradation product, which have androgenic properties (Rosa-Molinar and Williams, 1984; Howell and Denton, 1989). Furthermore, pulp and paper mill effluents can exert both estrogenic and anti-estrogenic activity in mammalian cells *in vitro* (Balaguer et al., 1996). As well, the livers of BKME-exposed white sucker rapidly accumulate ligands for the estrogen receptor, androgen receptor, and sex steroid binding protein (Hewitt et al., 2000). Although the situation is complex, the reproductive effects and hormonal changes indicate that constituents of pulp and paper mill effluents disrupt endocrine function in of exposed fish.

Contrasting effects of BKME have been observed on the secondary sexual characteristics of female mosquitofish (*Gambusia affinis*) in streams below paper mills in Florida, USA. These fish showed strong masculinization of the anal fin, which developed into a malelike gonopodium (Howell et al., 1980; Bortone and Cody, 1999). In males, the development of the anal fin into a gonopodium is under androgenic control. Therefore, it was suggested that the female mosquitofish had been exposed to androgenic compounds found in the effluent. Recent studies by Parks et al. (2001) determined that pulp mill effluent from a Florida mill contained compounds that exhibited androgen agonistic activity at levels

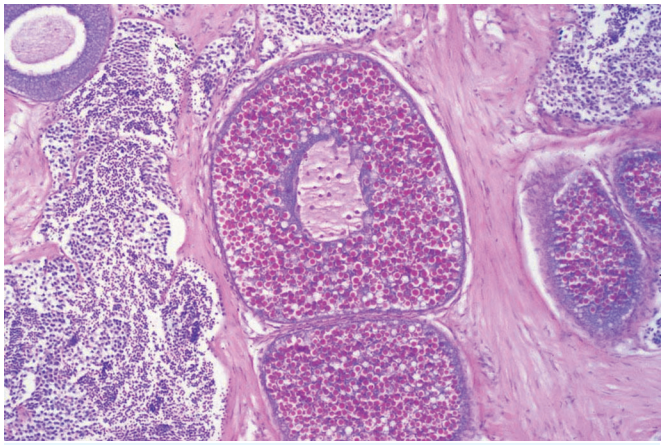


Figure 4.4. Testis section from a male flounder (*Platichthys flesus)* sampled in the U.K. Mersey estuary in 1996, showing several large secondary oocytes alongside abnormal sperm cell tissue (ovotestis). Reproduced by permission of the United Kingdom Centre for Environment, Fisheries, and Aquatic Science (CEFAS).

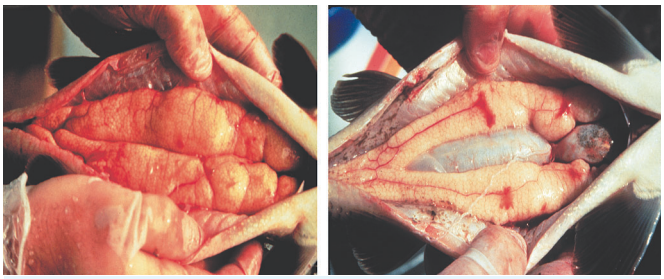


Figure 4.5. Ovaries from white sucker (*Catostomus commersoni*) collected during their spawning migration. The fish on the left was from a population living in a remote undeveloped region of Lake Superior (Mountain Bay). The fish on the right was from a population living in Jackfish Bay, Lake Superior, which receives BKME from a mill at Terrace Bay, Ontario, Canada. Ovarian size was significantly reduced in fish originating from the BKME-exposed site. Pictures provided by Drs M. McMaster, K. Munkittrick, and G. Van Der Kraak.

sufficient to account for the masculinization of female mosquitofish. It is not known whether the differential effects of the BKME on fish in Florida and Canada are due to differences in species sensitivities or to different substances discharged in the BKME.

The mechanism(s) behind the effects of BKME on secondary sexual characteristics is not well understood. However, it is plausible that the demasculinizing effect of BKME on feral fish in Jackfish Bay may be due to the concomitant decrease in plasma androgen (11-KT) levels (Munkittrick et al., 1991). It is possible that the masculinizing effect of the effluent may involve the actions of phytosterols. Denton et al. (1985) and Howell and Denton (1989) showed that partly biodegraded mixtures of plant sterols (β-sitosterol, campestrol, and stigmastanol) were able to induce nonreversible masculinization of the anal fin in adult female mosquitofish but that undegraded sterols were inactive. The precise mechanisms of action of these degraded sterols are poorly understood, but the production of androstenelike compounds may interfere at a site within the pituitary-gonadal axis or act agonistically at the androgen receptor. The latter mode of action has been observed in adult female goldfish (*Carassius auratus*), which develop male secondary sexual tubercles after exposure to implanted 11-KT (Kobayashi et al., 1991).

4.5.2.2.3 Possible cases of reduced reproductive success. There are very few examples of wildlife being affected at the population level by exposure to EDCs. In fish, one of the best examples of possible effects of EDCs on reproductive success in the field involves the PAH-and PCB-contaminated areas of Puget Sound on the west coast of the USA (Collier et al., 1998). A series of elegant studies on several flatfish populations including rock sole (*Pleuronectes bilineatus;* Johnson et al., 1998), winter flounder (*Pleuronectes americanus;* Johnson et al., 1992), and English sole (*Parophrys vetulus;* Johnson et al., 1988, 1997; Casillas et al., 1991; Landahl et al., 1997) have demonstrated a range of reproductive effects such as precocious female maturation, inhibited ovarian development, reduced egg weight, reduced spawning success, and reduced larval survival. The precise mechanisms behind these effects are not well understood, but they have been correlated with elevated levels of PAHs, DDT/DDE, and PCBs. The majority of the effects (especially reduced fecundity and spawning success) may be due to the anti-estrogenic effects of some PCBs and PAHs, or the interactions of PAHs or dioxins with the AhR. Stein et al. (1991) demonstrated that English sole injected with extracts of sediments from Puget Sound had reduced plasma E_2 levels. The estrogenic effects of various OCs may contribute to the precocious female maturation observed in English sole (Collier et al., 1998; Johnson et al., 1997). However, nonendocrine modes of action also need consideration due to the large number of contaminants present in the Puget Sound area.

4.5.2.2.4 Less-developed case studies. There are several less-developed examples suggesting that exposures to putative EDCs are affecting reproductive success in fish. For example, reduced production of perch (*Perca fluviatilis*) fry has been associated with endocrine-disrupting discharges of BKME in Sweden (Neuman and Karås, 1988; Sandström et al., 1988). Furthermore, reduced hatching success, reduced embryo and larval survival, and slower rates of development in fry have been reported in Atlantic herring (*Clupea harengus;* Hansen et al., 1985), Atlantic cod (*Gadus morhua;* Petersen et al. 1997), and European flounder (Von Westernhagen et al., 1981) from the Baltic Sea, lake trout (*Salvelinus namaycush*) from the Great Lakes (Mac and Edsall, 1991; Mac et al., 1993), and Arctic char (*Salvelinus alpinus*) from Lake Geneva (Monod, 1985).

Cases of possible but unconfirmed endocrine disruption–related impacts on gonad development by potential EDCs are numerous and include reduced and delayed ovarian development in flatfish after the *Amoco Cadiz* oil spill in Brittany (Stott et al., 1983), decreased egg weight and increased atresia in flatfish from contaminated harbors in the eastern USA (Johnson et al., 1992), and premature maturation in flatfish from the southern North Sea (Rijnsdorp and Vethaak, 1997) and Puget Sound (Collier et al., 1998; Johnson et al., 1997). These examples could be due to a range of possible endocrine-mediated mechanisms (including receptor-mediated effects, effects on the pituitary-gonadal pathway, or interference with steroid metabolism), yet nonendocrine toxicity and other environmental factors cannot be ruled out.

4.5.2.3 Altered sex steroid levels. In addition to the effects described above, there are several reports of reduced levels of sex steroids in both male and female fish exposed to phytoestrogens and BKME. For example, female white sucker exposed to BKME during vitellogenesis (period of gonadal development) in Jackfish Bay, Lake Superior, showed reduced levels of circulating E_2 and testosterone (Munkittrick et al., 1991, 1994; McMaster et al., 1991), and reduced testosterone and 17α,20β-P during the prespawning and spawning period (McMaster et al., 1991; Van Der Kraak et al., 1992) compared with levels of steroids found in female white suckers at reference locations. Similar depressions of 11-KT, testosterone, and 17α,20β-P were found in male white suckers. Also, longnose sucker and lake whitefish collected in the vicinity of Jackfish Bay (Munkittrick et al., 1992a, 1992b), other fish species from additional BKME-exposed sites in North America (Hodson et al., 1992; Gagnon et al., 1994; Adams et al., 1992), and perch and roach exposed to pulp mill effluents in Sweden and Finland have been reported to have depressed hormonal levels (Larsson et al., 1997; Van Der Kraak et al., 1997; Karels et al., 1999). A similar syndrome has been seen in BKME-exposed mummichogs (*Fundulus heteroclitus*; Dubé and MacLatchy, 2000). In laboratory experiments, fathead minnows exposed to BKME over their life cycle exhibit a parallel range of effects on circulating steroids and reproductive end points (Robinson, 1994; Kovacs et al., 1995). Together, these studies provide evidence that the reproductive responses were associated directly with effluent exposure and were not the result of other environmental factors such as habitat alteration.

Multiple locations in the hypothalamus-pituitary-gonad axis appear to be affected by constituents in BKME with endocrine-disrupting properties. During their spawning migration in Jackfish Bay, white sucker were found to have altered pituitary function, as determined by the depressed levels of plasma gonadotropin hormone (GtH-II) in males and females that were approximately 30–50-fold less than levels detected in control fish from a reference location (Van Der Kraak et al., 1992). In addition, exposed fish had a diminished response to a gonadotropin-releasing hormone analog (as determined by GtH levels), and *in vitro* studies showed that ovarian steroid biosynthetic capacity (i.e., testosterone and 17α,20β-P) was reduced. Finally, BKME-exposed fish had depressed levels of glucuronidated testosterone, which indicates that peripheral steroid metabolism was altered. Within the steroid biosynthetic pathway of the gonads, the predominant effect of BKME occurs downstream of pregnenolone (the immediate metabolite of cholesterol) (McMaster et al., 1995) and may inhibit the steroidogenic enzyme 17α-hydroxylase/C_{17-20} lyase.

The constituents of BKME that are responsible for, or contribute to, the endocrine disruption observed in exposed fish

have not been conclusively identified. The weakly estrogenic phytosterol, β-sitosterol is one compound known to be present in BKME that has been investigated as a possible EDC. In laboratory studies, goldfish exposed to β-sitosterol exhibit many of the reproductive effects observed in feral fish populations exposed to BKME, such as depressed levels of testosterone, 11-KT, and E_2 (MacLatchy et al., 1997). Other studies have reported that β-sitosterol does not act at the level of the estrogen receptor; rather, it interferes with the availability of cholesterol and pregnenolone (possibly via effects on cAMP formation), which impairs the steroid biosynthetic capacity of the gonads (MacLatchy and Van Der Kraak, 1995; Tremblay and Van Der Kraak, 1999). It is obvious that further work is necessary to identify the potential EDCs that are present in BKME and to discern how they elicit their effects on reproductive processes in fish.

Several other examples of altered levels of circulating sex steroids in fish have been noted in the field. For example, Folmar et al. (1996) found the male carp (*Cyprinus carpio*) collected near estrogenic STW discharges in the USA exhibited reduced levels of testosterone but not E_2. Elevated E_2/11-KT ratios were reported in wild carp that contained levels of pesticide residues that were above normal (Goodbred et al., 1997). Decreased plasma GtH and E_2 levels, but elevated ovarian testosterone production, were correlated with increasing body burdens of OCs in female kelp bass (*Paralabrax clathratus*) in the South California Bight (Spies and Thomas, 1997). Female European flounder held for 3 years on harbor sediment that was contaminated predominantly with PCBs and PAHs showed elevated testosterone and E_2 levels, which may have been the result of decreased steroid clearance via the CYP450 system (Janssen et al., 1997). Furthermore, male European flounder from estrogen contaminated U.K. estuaries contain elevated titers of E_2 (Scott et al., 2000). Of all the examples of altered sex steroid levels in fish from field studies, the case of BKME is the best understood.

4.5.2.4 Altered adrenal physiology. In fish, as in other animals, stress results in the activation of the HPI axis, culminating in increased plasma cortisol concentrations. There is now evidence from field studies that environmental contaminants may chronically stress fish resulting in a compromised HPI response. For example, Hontela et al. (1992, 1995) demonstrated that yellow perch (*Perca flavescens*) and northern pike (*Esox lucius*) from Canadian sites contaminated with PAHs, PCBs, and heavy metals were unable to produce cortisol in response to acute handling stress, and their ACTH-producing cells (i.e., pituitary corticotrophs) were atrophied. It was speculated that the atrophy was the result of prolonged hyperactivity of these cells. Consequences of this might involve an impaired ability of the fish to regulate energy metabolism. It was also suggested that the clearance rate of cortisol might have been increased by the contaminant-induced CYP450 mixed-function oxidase system. In both sexes of yellow perch, gonad size and thyroxine levels were reduced. Other studies by Hontela et al. (1997) showed that the effects of BKME were similar to the effects of PCBs, PAHs, and heavy metals. In these studies, both the corticotrophs and the interrenal steroid–producing cells were atrophied.

Recent work by Norris et al. (1997b, 1999) showed that brown trout (*Salmo trutta*) living in metal-contaminated waters in the USA had comparable levels of cortisol compared with control fish (both groups of fish were stressed by electroshocking). Yet, the fish exposed to metals were found to be hypersecreting ACTH and corticotropin-releasing hormone to maintain baseline levels of cortisol. Similar studies demonstrated that fish chronically exposed to metal contaminants could not maintain ACTH and cortisol levels compared with controls in acute stress trials (Norris et al., 1999). Another report by Girard et al. (1998) showed that yellow perch from contaminated sites synthesize less cortisol in response to an injection of ACTH compared with fish collected from control sites. This indicates that damaged interrenal tissue contributes to the observed effects, because ACTH could not alleviate the impaired cortisol response. More research is necessary to determine the extent of EDC-induced compromised stress responses in fish, as well as its potential impacts on the health of populations.

4.5.2.5 Early-life-stage mortality. Contaminant-induced endocrine disruption may be responsible for blue-sac disease in Great Lakes salmonids, which is a condition characterized by yolk sac edema, regional ischemia, hemorrhaging, craniofacial abnormalities, and mortality early in larval development (Symula et al., 1990; Cook et al., 1997; Ankley and Giesy, 1998; see Figure 4.6). Probably the most developed cases involve lake trout, but similar conditions have been reported in several species of *Salvelinus*, *Oncorhynchus*, and *Salmo*. This condition has been reproduced in laboratory studies using TCDD (and related compounds), as well as extracts of adult lake trout from Lake Michigan, which contained PCDDs, PCDFs, and PCB congeners (Giesy and Snyder, 1998). A retrospective analysis (Giesy and Snyder, 1998) of the incidences of blue-sac disease and exposure to these contaminants found a relationship between observed trends in lake trout reproduction in Lake Ontario. It concluded that, in the past, AhR agonists were primary contributors to the adverse population level impacts in this

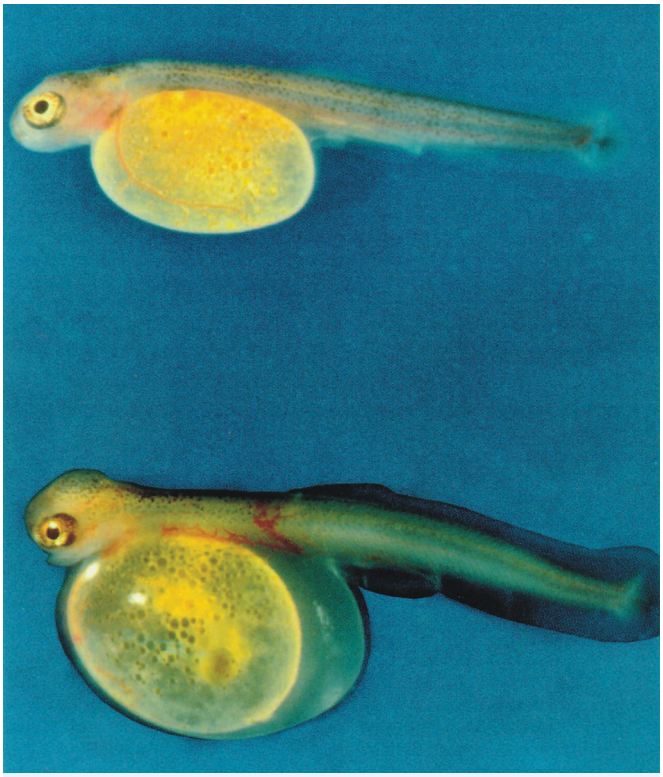


Figure 4.6. Lake trout (*Salvelinus namaycush*) sac fry exposed as a fertilized egg to control vehicle (top) or to TCDD in vehicle (bottom). The bottom fry shows signs of blue-sac disease, including yolk sac and pericardial edema, subcutaneous hemorrhages, and craniofacial malformations. Reprinted from Cook et al. (1997).

system. However, possible mechanistic linkages between AhR agonists and endocrine disruption are poorly understood.

Another condition noted in 1968 to the present in Great Lakes fish was EMS (Marcquenski and Brown, 1997). This syndrome affects the fry and is characterized by a loss of equilibrium, spiral swimming, lethargy, hemorrhage, and death. An analogous condition occurs in Atlantic salmon (*Salmo salar*) inhabiting the Baltic Sea and is termed M74 (Börjeson and Norrgren, 1997; Bengtsson et al., 1999). Although the exact cause of EMS and M74 in the wild is unknown, diet may play a role. Laboratory experiments have shown that both EMS and M74 are caused by thiamine deficiency and could be related to differential thiamine or thiaminase contents in prey species.

There are data suggesting that contaminants may play a role etiology of EMS and M74. For example, Tillitt et al. (1996) showed that thiamine can protect against the deleterious effects of dioxin to fish embryonic development, so a natural thiamine deficiency could exacerbate the effects of OC contamination. Also, there seems to be a correlation between incidences of M74 and elevated body burdens of PCDFs and coplanar PCBs (Vuorinen et al., 1997a; Vuorinen and Keinänen, 1999). Another mechanism that has been suggested is that M74 may be the result of toxicant-induced alterations in thyroid hormone and retinol levels, but the evidence is weak (Vuorinen et al., 1997b). Both syndromes have not been conclusively linked to exposure to EDCs, nor has an endocrine-based mechanism been demonstrated.

The deaths of juvenile salmon in Canadian river basins exposed to the insecticide Matacil 1.8D provide another example of possible endocrine disruption in feral fish. Matacil 1.8D is formulated with NP. Fairchild et al. (1999) showed that a significant proportion of the lowest Atlantic salmon catches, and heavy salmon smolt mortality, coincided with spraying of Matacil 1.8D for spruce budworm control. A similar product that is formulated without NP (Matacil 1.8F) did not have these effects. Low catches of blueback herring (*Alosa aestivalis*) also coincided with the spraying of Matacil 1.8D. Fairchild et al. (1999) estimated that the spraying of the insecticide would have resulted in sufficient concentrations of NP in the river water to cause estrogenic effects in the fish. Furthermore, Madsen et al. (1997) demonstrated in the laboratory that NP could inhibit smoltification and impair hyposmoregulation in salmon. It is known that sex steroids and the entire process of sexual maturation antagonize the physiological processes of smoltification and seawater acclimation. However, the precise mechanisms involved and the actions of NP on these processes are not well understood.

4.5.2.6 Thyroid dysfunction. Salmonid species in the Great Lakes have been found to suffer from abnormally high incidence of thyroid dysfunctions (i.e., goiter). Although enlargement of the thyroid gland in vertebrates can be due to a natural iodine deficiency in the diet, this has been ruled out as a causative factor in the case of these salmonids (Leatherland, 1994). Depressed thyroid hormone levels have been observed in many salmonid species in the Great Lakes, particularly Lake Michigan and Lake Erie (Leatherland and Sonstegard, 1980, 1984; Leatherland et al., 1989). It was originally suspected that OCs, functioning as environmental goitrogens, might be responsible. Laboratory studies of salmon chronically dosed with PCBs or perchlordecone did exhibit lowered thyroid hormone levels (Leatherland and Sonstegard, 1978). However, feeding trials in which rainbow trout and coho salmon (*Oncorhynchus kisutch*) were fed OC-contaminated fish from the Great Lakes failed to produce the same effects (Leatherland, 1992, 1993; Leatherland and Sonstegard, 1982). Nevertheless, goiters and depressed serum thyroid hormone levels were produced in rodents fed contaminated fish (Leatherland, 1992, 1993), indicating the presence of unidentified goitrogens. It is still not known which compounds are affecting thyroid hormone economy in Great Lakes fish, but there is recent evidence (Leatherland, 1993, 1994) that environmental factors other than EDCs may be responsible for the effects.

4.5.3 Conclusion

This brief review shows that the endocrine disruption that is undoubtedly occurring in wild fish populations in North America, Asia, Australia, and Europe is caused through a variety of mechanisms including hormone receptor interactions, interference with the biosynthesis of sex steroids, and perturbations of the hormonal control by the pituitary on reproductive and adrenal processes. However, in most cases the precise modes of action are still poorly understood, and data are largely confined to the gonochoristic species. The compounds responsible for the observed effects may be due to both synthetic and natural compounds. Currently, there is limited understanding of how the existing endocrine disruption affects population fitness.

4.6 Invertebrates

4.6.1 Unique Aspects

Most major contemporary groups of invertebrates are the product of an evolutionary lineage distinct from that of the vertebrates. Accordingly, the endocrine systems of most vertebrate groups share little with vertebrates, and invertebrates exhibit unique susceptibilities to EDCs. Invertebrates also exhibit a variety of anatomical and physiological traits that are under endocrine control and are not present in vertebrates. For example, many invertebrates have complicated life histories and display various forms of hermaphroditism. Some species also exhibit poorly defined sexual dimorphism. Reproductive cycles can be highly complex involving environmental stimuli, such as light intensity, temperature, desiccation and diet. Also, there are unique aspects of sex determination to be considered in certain species. The sex of an individual is not always determined before or at fertilization but may be influenced by environmental conditions experienced during embryonic or larval development (Stahl et al., 1999). For example, in the echiuran worm *Bonellia viridis,* females release a substance that masculinizes developing larvae. The absence of this substance results in the production of female larvae. Separating the effects of various environmental stimuli from the potential effects of EDCs will pose a significant challenge in many invertebrate species. More conventional regulation of sex differentiation and gonadal development by hormones also occurs in some invertebrates, although the hormone involved differs from those of vertebrates. Environmental compounds with endocrine-disrupting properties may influence these hormonally regulated processes, as in vertebrate wildlife.

Consistent with the function of the vertebrate endocrine system, the endocrine system of invertebrates functions to transduce signals, either environmental or endogenous, to appropriate target sites in order to elicit the required response (LeBlanc et al., 1999). These signaling pathways are often initiated with neuropeptides that may directly stimulate the targeted response or may be part of a cascade of hormones, which include nonpeptide hormones such as steroid hormones and juvenoid hormones. As in vertebrates, these nonpeptide hormones may be more susceptible to endocrine toxicants because exogenous chemicals are more likely to interact with the receptors to these hormones.

Two major classes of hormones in the arthropods (i.e., crustaceans, insects, and some minor phyla) are the ecdysteroids and juvenoids. These hormones share significant structural and functional homology with the sex steroids and retinoids of vertebrates. Ecdysteroids are primarily recognized for their regulation of molting, embryo development, diapause, cuticle formation, and various aspects of reproduction including VTG production, ovulation, and spermatogenesis (Hagedorn, 1985; Koolman, 1989). Terpenoids (specifically the juvenoids) have been recognized primarily in insects for their role in promoting juvenile to adult metamorphosis (LeBlanc et al., 1999). Terpenoids are now recognized to function in concert with, and perhaps independently of, ecdysteroids to regulate a variety of functions including reproduction, caste determination, behavior, diapause, and metabolism (Nijhout, 1998). The action of both ecdysteroids and retinoids is mediated by specific receptors that are likely susceptible to binding by EDCs.

Unique lifestyle and habitat preferences of many invertebrates are influential factors in determining the extent of their exposure to EDCs. Filter-feeding organisms are exposed to EDCs through the water phase (and the particulate components in water), and deposit feeders encounter EDCs adsorbed to sediment particles (Depledge and Billinghurst, 1999). For example, deposit feeding organisms like the polychaete worm *Dinophilus gyrociliatus* may be affected by NP, which accumulates on particulate matter. Controlled experiments demonstrated that exposure of *D. gyrociliatus* to environmentally realistic concentrations of NP was associated with increased egg production but reduced dgg viability (Price and Depledge, 1998). Herbivorous species potentially ingest a wide range of natural phytoestrogens and mycoestrogens. Interestingly, some phytosteroids are highly soluble in water and are structurally similar (brassinosteroids) to ecdysteroids (the arthropod molting hormone) (Luu and Werner, 1995). Indeed, Subramanian and Varadaraj (1993) found that molting is arrested in some aquatic insects exposed to paper and pulp mill effluents that contain high concentrations of phytosteroids. As well, carnivorous invertebrates may consume significant amounts of hormone mimics from contaminated prey.

4.6.2 Effect-Based Responses and Case Studies

4.6.2.1 Effects of TBT on gastropods and bivalves. The most complete example of endocrine disruption by an environmental contaminant is documented in mollusks exposed to TBT, a compound found in antifouling paints that are applied to the hulls of ships. In the 1980s, the condition of imposex (the imposition of male sex organs including a penis and vas deferens onto females) was observed with increasing frequency in marine gastropods exposed to TBT (Bryan et al., 1986; Smith and McVeagh, 1991; see Figure 4.7). Supporting work demonstrated that injections of TBT into female common dogwhelks (*Nucella lapillus*) induce penis formation (Lee, 1991; Spooner et al., 1991). The frequency of imposex and degree of penis development are related to the degree of TBT exposure (Bryan et al., 1986). The consequences of imposex in the dogwhelk *Nucella lapillus* include distortions of the sex ratio, reductions in the recruitment of juveniles, and reduced population numbers (Matthiessen et al., 1999). Approximately 150 different species of prosobranch gastropods have been affected by organotins (TBT and triphenyl tin) worldwide (Matthiessen et al., 1999). TBT also induces a second masculinization phenomenon in the periwinkle *Littorina littorea*: intersex (Oehlmann, 1998). In this case, the female pallial organs are modified toward a male morphological structure that can lead to the formation of a prostate gland. Both imposex and intersex can result in the sterilization of the females.

Testosterone and the peptide APGW-amide have been shown to stimulate male sex organ development in female gastropods (Bettin et al., 1996; Oberdorster and McClellan-Green, 2001). These hormones may function to stimulate male sex organ development. Currently, TBT is not known to influence the activity of APGW-amide; however, TBT does elevate testosterone levels in female gastropods (Spooner et al., 1991; Bettin et al., 1996; Matthiessen and Gibbs, 1998). The inhibition of testosterone aromatization or conjugation of testosterone to polar, highly excretable derivatives of testosterone has been proposed as possible mechanisms by which TBT elevates testosterone levels (Spooner et al., 1991; Stroben et al., 1991). Recent studies using the mug snail (*Ilyanassa obsolete*) have revealed that testosterone is conjugated primarily to fatty acids that are retained by the snails (Gooding and LeBlanc, 2001). This is a rather unique mode of testosterone inactivation, and the TBT-induced inhibition of the acyl coenzyme A:testosterone acyltransferase responsible for this esterification may prove to be the reason why gastropods are uniquely susceptible to the endocrine-disrupting activity of TBT.

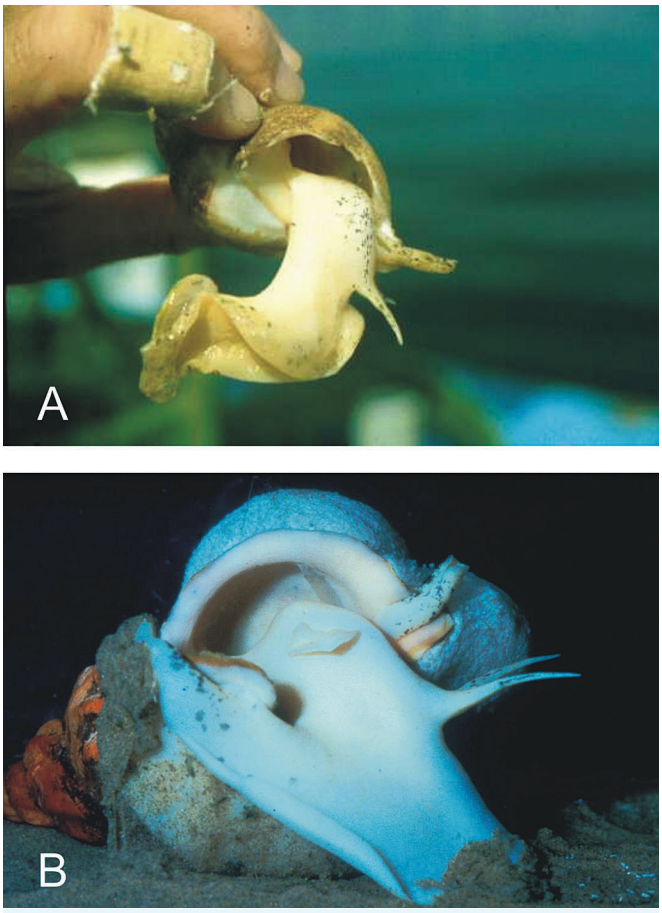


Figure 4.7. Imposex in the common whelk (*Buccinum undatum* L). *A*) A female whelk without imposex: the head and tentacles (to the right) and the body are smooth (extended foot shown to the left lower side of the picture). *B*) A female with imposex: a skin protrusion has developed at the right side of the body, below the right tentacle; this is a sign of imposex. Pictures provided by Dr C.C. ten Hallers-Tjabbes, H. Kralt, and Dr J.P. Boon, Royal Netherlands Institute for Sea Research.

In addition to gastropods, TBT has been found to have various effects on the reproduction of several species of bivalve mollusks, such as the European flat oyster (*Ostrea edulis*), blue mussels (*Mytilus edulis*), and an intertidal clam (*Scrobicularia plana*) (Matthiessen et al., 1999). The Pacific oyster (*Crassostrea gigas*) develops unusual shell morphology with thickened valves and internal chambers in response to TBT exposure, and in Arcachon, France, and in the United Kingdom, shell deformities were found to be less severe farther from marinas (Alzieu, 1991). At high concentrations, female oysters exhibit altered reproductive systems and spermatogenesis may occur. Although reproductive effects of TBT in bivalves have been linked to alterations in endocrine function, it is unclear whether shell thickening in Pacific oysters is a consequence of endocrine disruption (Matthiessen et al., 1999).

4.6.2.2 Endocrine disruption in crustaceans.

4.6.2.2.1 Disruption of ecdysteroid-regulated processes. Ecdysteroids elicit physiological responses through interaction with an ecdysone receptor. Environmental chemicals have the potential to function as ecdysone agonists by binding to and activating the ecdysone receptor. Many plant compounds are known to function as ecdysone receptor agonists (Dinan et al., 2001a, 2001b), and some insecticides function by acting as ecdysteroids (Sundaram et al., 1998). Ecdysteroid agonists have been shown to accelerate molting, cause incomplete ecdysis, and cause death during molting (Clare et al., 1992; Baldwin et al., 2001).

Environmental chemicals also can bind to the ecdysone receptor in an antagonistic fashion. Chemicals shown to function as ecdysone receptor antagonists include bisphenol A, lindane, and diethylphthalate (Dinan et al., 2001a, 2001b). The fungicide fenarimol has been shown to function as an antiecdysteroid in crustaceans by lowering levels of endogenous ecdysone (Mu and LeBlanc, 2001). Consequences of antiecdysteroids exposure to crustaceans include delayed molting and developmental abnormalities (Mu and LeBlanc, 2001). The steroidal androgens testosterone and androstenedione have been shown to elicit antiecdysteroidal activity, and this property is likely responsible for the developmental toxicity of these chemicals to crustaceans (LeBlanc et al., 2000; Dinan et al., 2001a).

4.6.2.2.2 Disruption of juvenoid-regulated processes. The structure and function of juvenile hormones have been thoroughly characterized in insects. These hormones appear to function through interaction with retinoid X-like receptors (i.e., ultraspherical) that heterodimerize with the ecdysone and perhaps other receptors (Yao et al., 1993; Jones and Sharp, 1997). In crustaceans, methyl farnesoate has functional homology to the juvenile hormones of insects (LeBlanc et al., 1999). Methoprene is a juvenile hormone analog that elicits a variety of effects in crustaceans that are suggestive of endocrine disruption. Methoprene has been reported to reduce fecundity (Templeton and Laufer, 1983; McKenney and Celestial, 1996; Chu et al., 1997; Olmstead and LeBlanc, 2001a), interfere with juvenile development (Templeton and Laufer, 1983; Celestial and McKenney, 1994), reduce growth rate and molt frequency (Olmstead and LeBlanc, 2001a), and delay reproductive maturation (Olmstead and LeBlanc, 2001a). Methoprene also has been shown to stimulate excess male production in *Daphnia magna* (Olmstead and LeBlanc, 2001b). Many of these effects occurred at methoprene levels significantly lower than environmental concentrations measured at application (Knuth, 1989). Environmental chemicals such as atrazine and 4-NP also have been shown to stimulate male production in daphnids (Dodson et al., 1999a), whereas dieldrin exposure reduced the number of males produced by daphnids (Dodson et al., 1999b). These observations raise the possibility that environmental chemicals may interfere with juvenoid-regulated processes in crustaceans with adverse consequences.

4.6.2.3 Molting disturbances and deformities in insects. There is a large body of literature on the effects of certain chemicals on the insect endocrine system due to the development of insecticides that are designed to alter endocrine functioning. Endocrine-disrupting effects of insecticides on nontarget insects have also been reported. For example, treatment of orchards with fenoxycarb (a juvenile hormone analog) has been shown to have deleterious effects on the development of honeybee broods (LeBlanc et al., 1999).

The ecdysteroid receptor agonist tebufenozide causes hyperecdysonism in certain insects (Wing, 1988). These effects include the delayed postembryonic development of the diamondback moth (*Plutella xylostella* L.) and the gray fleshfly (*Neobellieria bullata* Parker). Also, tebufenozide induces molting disturbances in Colorado potato beetle (*Leptinotarsa decemlineata* Say) and cabbage butterfly (*Pieris brassicae* Hubner) and causes an early first molt in *N. bullata* and nymphal-adult intermediates in the milkweed bug (*Oncopeltus fasciatus* Dallas). In the latter species, exposure resulted in sterility (Darvas et al., 1992). Darvas also found wing and mandible malformations of insects following exposure to tebufenozide.

Phytoectosetroids have been reported to have hormonal activity in aquatic insects (Adler and Grebenok, 1995). Effluents from pulp and paper mills, which contain phytoectosetroids, have been found to influence molting in dragon fly larvae (Subramanian and Varadaraj, 1993). Exposure of the same species to tannery effluent resulted in a shortened time to first molt. The authors suggested that components of the effluents might have interfered with ecdysteroid metabolism.

In chironomid larvae, increased incidences of deformities of the mouthparts and other head capsule features may be induced by exposure to sediments contaminated with DDE, heavy metals, and pulp and paper mill effluents (Matthiessen et al., 1999). In controlled laboratory studies, chironomid larvae deformities were induced by exposure to heavy metal concentrations similar to those found in the field (Dickman and Rygiel, 1996). Although the underlying mechanism responsible for these effects are unknown, the developmental alterations that occur have led to the hypothesis that an endocrine-based mechanism is involved.

4.6.3 Conclusion

The diversity of the invertebrate phyla creates numerous challenges in determining the potential risks of EDCs to the health of these animals. Compounding these challenges is the poor understanding of the endocrinology of most invertebrates, and even though the endocrinology of arthropods is best understood among invertebrates, gaps in our knowledge still exist. It is clear that the effects of EDCs in vertebrates will not necessarily be similar to those observed in invertebrates. Conversely, invertebrates are susceptible to endocrine-disrupting properties of compounds that are not problematic in vertebrates. More field-based studies are required to determine the extent to which invertebrate species, both aquatic and terrestrial, have been impacted by exposure to EDCs in their environment.

4.7 Uncertainties and Research Needs

The case studies reported in this chapter have provided strong evidence that there are effects observed in wildlife that can be attributed to substances that function as EDCs. However, there are a

large number of situations where the evidence of a causal link with endocrine disruption is weak or nonexistent. It is apparent that the variety of responses in wildlife that can be attributed to EDCs are most obvious in species inhabiting areas that have received extensive chemical contamination. There are unanswered questions regarding whether areas contaminated with lower levels (i.e., background levels) of compounds also pose significant risks to wildlife, or whether the ranges of effects are restricted to the wildlife studied this far.

The case studies illustrate some of the challenges encountered when establishing cause-and-effect relationships between chemical exposures and physiological dysfunction in diverse species. All ecotoxicological investigations are complicated by a variety of factors that may impact growth, reproduction, and survival. For example, food availability, disease state, competition, and loss of habitat are significant stressors to wildlife and impinge on many of the end points measured in investigations that assess the risks posed to wildlife by EDCs. Other confounding factors relate to the lack of knowledge of the endocrine, reproductive, and developmental biology in many wildlife species. Although it is recognized that there are critical stages of embryonic developmental that may be especially impacted by EDCs, few studies have explored this issue. Given the diversity of wildlife, it may be inappropriate to extrapolate the responses to EDCs and other stressors, because research has focused predominantly on only a few species. Currently, routine ecotoxicological assessments tend to focus only on a few species of wildlife. This approach also limits the ability to assess the ecological relevance of anthropogenic influences on the environment. As an example, one group of avian species that has been underrepresented is passerine birds. This has occurred despite evidence of widespread declines in populations of several species of songbirds. Similarly, there have been few studies that have considered invertebrates despite the knowledge that invertebrates are key to the structure and function of ecological systems.

Most ecological risk assessments of wildlife tend to focus on populations and communities. However, when considering the potential effects of EDCs to wildlife, the focus tends to be on the individual. This may be problematic because there is a limited understanding of how physiological changes affect the individual or how individual responses affect population and community outcomes. The ecological significance of disturbances in growth, reproductive output, viability of offspring, altered sex ratio, and of potential transgenerational effects is difficult to quantify. One area of investigation that merits consideration is the possibility that artificial selection has occurred as a result of multigenerational exposure to EDCs.

Compared with common laboratory models, many wildlife species are difficult to sample in a systematic fashion due to their lifestyle. To determine the extent of EDC-induced effects (population decline, malformations) on some species of wildlife, long-term monitoring is needed that would provide baseline data on population status. This type of research would be instrumental when attempting to delineate the relative risks of EDCs from other stressors and for perspective analyses associated with the release of new chemicals in the environment.

Given the breadth of responses seen in wildlife to date, it is imperative that research continues to address the extent of risk posed to wildlife by EDCs, although, as far as ecological risk assessments are concerned, discussion of the "endocrine disruptor hypothesis" is somewhat of an artificial classification. The critical issue is to assess the status of wildlife populations in terms of possible effects on growth, reproduction, and survival. This will necessitate collaboration and cooperation on an international scale to identify populations that are at risk, to dedicate the resources to address critical knowledge gaps, and to ensure that the most current and up-to-date methodologies in endocrinology, physiology, ecology, and epidemiology are used when evaluating effects on wildlife. Finally, we need to communicate the results of studies widely and effectively such that the true risks posed by EDCs are understood and that policy decisions and regulatory practices are based on the best scientific knowledge.

A variety of human health concerns have been raised in relation to endocrine disruptors. Attention has focused on health end points considered to be potentially at risk because either their development or their later functioning in adult life is known or thought to be influenced by exposure to chemicals with endocrine activity.

In this chapter, four main areas are reviewed: reproduction, neurobehavior, immune function, and cancer. The order of the chapter sections reflects the continuum of life from conception through adult life. Because sex hormones and thyroid hormones are major determinants of development and function of the reproductive, central nervous, and immune systems, much of the experimental research to date has focused on the effects of EDCs on these key hormone systems and their target tissues, and possible links to human health effects. Endocrine-mediated mechanisms are well established for certain human cancers, and these are also addressed.

Although a few examples in this chapter clearly demonstrate adverse effects in humans after high exposures to environmental chemicals (such as poisoning incidents), the data are much less clear for lower levels of exposure, either to single chemicals or to mixtures with potentially similar actions. It is these lower levels of exposure that raise the most important public health questions, because even small shifts in population distributions of adverse health outcomes, such as infertility or lowered IQ, could potentially have considerable impact on the overall health of large populations. The inherent problems in comparing human, laboratory, and epidemiological studies conducted at different times and locations and under different conditions continue to affect our ability to draw firm conclusions about the existence of any global disease trends, and the lack of adequate exposure data severely hampers the exploration of hypotheses regarding possible causes of any identified trends. Similarly, the lack of exposure data during critical periods of development that influence later functioning in life make it difficult to draw causative associations between exposure and effect.

Hence, this chapter not only explores the available human health data but also draws on relevant data from experimental studies in laboratory animals where they support or cast doubt on the biological plausibility of an adverse effect on human health from EDCs. This chapter is not intended to be a comprehensive review of the experimental literature, which has grown exponentially in the last decade. However, it is only by integrated consideration of the background endocrinology, the human data, and the animal findings that the strength of the endocrine disruptor hypothesis can be evaluated.

5.1 Reproduction

5.1.1 Introduction

The possibility that environmental exposure to chemicals might affect human reproduction is not new. However, the hypothesis that environmental chemicals acting as EDCs could be causative agents of changes in population-based, reproductive health trends is relatively recent. Changes occurring in various human reproductive health statistics, particularly changes in temporal and geographical trends, play a key role in the debate about possible effects of exposure to EDCs. Although the main focus to date has been on male reproductive health (Toppari et al., 1996), this review covers both male and female reproductive function.

Much of the interest in the male reproductive system has stemmed from an hypothesis proposed by Sharpe and Skakkebaek (1993) that agents that interfere with normal development of the reproductive system via an endocrine mechanism could plausibly be related to increases noted in human male reproductive disorders over a number of years. In particular, a link was made between developmental events that could result in decreased sperm count/quality and increased incidences of testicular cancer, testicular maldescent (cryptorchidism), and male reproductive tract malformations, such as hypospadias. These could be expressions of one underlying entity, the testicular dysgenesis syndrome (Skakkebaek et al., 2001), which could result from disruption of gonadal development during fetal life. Although the evidence linking such effects to human exposure to chemicals is very weak or nonexistent, treatment of experimental animals with certain chemicals during critical windows of development of the male reproductive system can result in deficits of the type proposed by the hypothesis (Chapter 3, section 3.12). The one major exception is the production of testicular cancer (section 5.4.4). Seminoma, the major

List of Abbreviations

2,4-D 2,4-Dichlorophenoxyacetic acid
AhR Aryl hydrocarbon receptor
AR Androgen receptor
CI Confidence interval
CIS Carcinoma *in situ*
d day
DBCP Dibromodichloropropane
DDE Dichlorodiphenyl dichloroethylene
DES Diethylstilbestrol
DDT Dichlorodiphenyl trichloroethane
DHT Dihydrotestosterone
DTH Delayed-type hypersensitivity
E_2 17β-Estradiol
EDCs Endocrine-disrupting chemicals
EGCG (–)-Epigallocatechin gallate
EGF Epidermal growth factor
ER Estrogen receptor (α and β isoforms)
FSH Follicle-stimulating hormone
GD Gestational day
GR Glucocorticoid receptor
HCB Hexachlorobenzene
HPOA Hypothalamic preoptic area
HRT Hormone replacement therapy
IARC International Agency for Research on Cancer
Ig Immunoglobulin
IPCS International Programme on Chemical Safety
LA Los Angeles
LH Luteinizing hormone
LOAEL Lowest observed adverse effect level
MRC Medical Research Council
NOAEL No observed adverse effect level
NYC New York City
OECD Organisation for Economic Co-operation and Development
OR Odds ratio
PBBs Polybrominated biphenyls
PCBs Polychlorinated biphenyls
PCDDs Polychlorinated dibenzodioxins
PCOS Polycystic ovary syndrome
PCDFs Polychlorinated dibenzofurans
PHAHs Polyhalogenated aromatic hydrocarbons
PND Postnatal day
RACB Reproductive assessment through continuous breeding
T_3 Triiodothyronine
T_4 Thyroxine
TCDD 2,3,7,8-Tetrachlorodibenzyl-*p*-dioxin
TEQs Toxic equivalent quotients
TGF Tumor growth factor
TPOs Thyroid peroxidase inhibitors
TSH Thyroid-stimulating hormone
USA United States of America
US EPA United States Environmental Protection Agency
US FDA United States Food and Drug Administration
wt weight

form of human testicular cancer, is extremely rare in laboratory animals, and until recently, no experimental model existed for its production (section 5.4.4.2). However, some chemicals have been shown to produce interstitial (Leydig) cell tumors, particularly in rodents, that have been related to disturbances in normal endocrine control of male reproductive function.

Changing trends in female reproductive health have been much less studied than those in males. However, female reproductive development is also susceptible to endocrine interference (Chapter 3), and effects on female reproductive tissues and pregnancy outcomes may be of value as indicators of environmental influences. A number of aspects of female reproductive health are discussed below, and breast cancer is discussed in section 5.4.2.

The following sections summarize the available literature on adverse outcomes in human reproductive health that have either been proposed to be due to exposure to endocrine disruptors, or for which an endocrine mechanism is plausible or has been demonstrated. A number of examples of chemical influences on human reproduction are cited, but it should be noted that evidence that these are clearly linked to endocrine-mediated effects is lacking. Emphasis has been put on the plausibility of the endocrine disruptor hypothesis in relation to each end point considered and on causality. Although the focus is on the human literature, experimental animal data have been included to explore plausibility issues and potential mechanistic pathways involving endocrine disruption.

5.1.2 Sperm Quality and Testis Function

5.1.2.1 Assessment of testis function. Spermatogenesis is the process whereby normal spermatozoa are produced from spermatogonia in the seminiferous epithelium of the testis. Initial mitotic divisions result in proliferation of the spermatogonial stem cells that will eventually enter meiotic prophase, where, as spermatocytes, the cells undergo a series of transformations and eventually reduction divisions to reduce the chromosome number by half and produce spermatids. These round cells then undergo a metamorphosis to produce the elongated spermatids that are eventually released from the epithelium to enter the lumen of the seminiferous tubule, thence to the rete testis, the efferent ducts, and epididymis, where the sperm acquire the ability to become motile and fertilize an ovum. All of these cellular changes are under close hormonal control at the endocrine, paracrine, and autocrine levels (Chapter 3) and thus are potential targets for the effects of endocrine-active chemicals, including (anti)estrogens (anti)androgens, steroid biosynthesis inhibitors, and a variety of growth factor mimics.

In animal species, all of these potential targets can be evaluated in standard bioassays and research study designs following treatment with chemicals, and clear relationships can be developed between internal target dose and tissue histology, cellular function, and biochemical end points. The ability to undertake such studies in human males is far more limited and is normally restricted to circulating hormone levels (which tend to be insensitive or reflect only serious deficits) or some measure of sperm quality (number, motility, morphology, function). Although these parameters can be measured in animals to enable a direct comparison with humans, the process for the production of sperm is far more efficient in laboratory animal species, and small perturbations have a lesser chance to induce functional deficits.

Potential fertility in the male is reduced when the sperm concentration is very low. However, there is no consensus on the value of this limit. From studies in couples attempting to conceive, it has been found that the fertility potential of men decreased when the sperm concentration was under 40×10^6/ml (Bonde et al., 1998). A similar figure of 48×10^6/ml has also been recently reported (Guzick et al., 2001).

Other sperm characteristics, such as the percentage of motile forms or the incidence and the type of morphological abnormalities, seem to be more directly related to male fertility potential. Recent data that attempt to define a "normal" semen evaluation indicate, for example, that sperm morphology is a better discriminator between fertile and infertile men than is sperm concentration (Guzick et al., 2001). Sperm morphology and motility could also be useful markers of toxic damage even in the absence of any effect on fertility. The fertilizing ability of spermatozoa is a complex phenomenon involving many molecular and cellular events. Some of them are essential, such as the kinetic function allowing the sperm to reach the oocyte, or capacitation and the acrosome reaction necessary for the interaction with the zona pellucida and the fusion with the oocyte plasma membrane. The nuclear composition and DNA integrity are also important factors for normal embryo development. Sophisticated biological tests have been developed in recent years to evaluate various sperm functions, but no single test can ideally measure the fertility potential of a man.

In practice, if the sperm count is used to compare fertility potential of different populations at different times or in different places, it may be more appropriate to analyze the distribution of sperm concentration and more especially the proportion of men with lower values instead of comparing the arithmetic means or even the medians or the geometric means. Sperm concentration is dependent on the volume of seminal plasma in that the germinal cells are diluted at ejaculation. Because the seminal plasma volume may vary, sperm count (concentration × volume) rather than sperm concentration may be a better quantitative marker of spermatogenesis, although both are used clinically. It is also useful to compare the percentage of motile and morphologically abnormal sperm. Alternatively, the concentration of motile sperm, as calculated by Irvine et al. (1996), may better reflect the fertility potential than does the sperm count. Although sperm characteristics such as the percentage of motile and morphologically normal sperm are simple and useful qualitative indicators of spermatogenesis, they are not as easy to measure as sperm count.

If sperm count is not the best indicator of male fertility potential, it is an interesting biomarker of testis function and more precisely of spermatogenesis and Sertoli cell number, size, and activity. There is a significant relationship between the number of Sertoli cells and the number of spermatozoa produced. Therefore, any factor that alters Sertoli cell multiplication and differentiation during testis development or irreversibly damages Sertoli cells postpuberty will reduce the number of spermatozoa produced by the testis.

5.1.2.2 Are there temporal and/or geographical trends in human sperm quality and testis function? The issue of whether particular regions show temporal declines in sperm count and testis function has been discussed over a long time, not just in relation to more recent concerns about EDCs. It has been hypothesized that there is a "global" or worldwide decline, but this is not supported by the information available to date.

A possible decline in human sperm quality was first suggested by Nelson and Bunge (1974), who reported a mean sperm concentration of 48×10^6/ml in 390 fertile men requesting a

vasectomy in Iowa City, which was lower than the then expected normal values. Rehan et al. (1975) reported that the mean sperm count was 79×10^6/ml among 1,300 fertile men requesting vasectomy in NYC. These results together with the data of a third American study (Zukerman et al., 1977) were reviewed by MacLeod and Wang (1979), who concluded, based on the data from infertile men attending their own laboratory over a 30-year period, that there were no convincing arguments in favor of a temporal decline in human sperm count. However, based on a meta-analysis of 17 articles published from 1934 to 1979 reporting the mean sperm counts of unselected men of proven fertility, James (1980) concluded that there was "good reason for supposing that there has been a decline in *reported* counts."

The question was highlighted again in a 1992 meta-analysis, based on 61 articles, which concluded that the mean sperm concentration of healthy men had declined from 113×10^6/ml to 66×10^6/ml within 50 years between 1938 and 1991 (Carlsen et al., 1992). The principal criticisms of the above meta-analysis were that the men included in several studies could have been very heterogeneous in terms of their geographic location, season of study, fertility, age, and socioeconomic conditions, so they could hardly be compared. Additionally, the sample sizes in many studies were very small and thus did not permit useful comparisons to be made (Farrow, 1994). It was also suggested that the results could be influenced by confounding factors or by differences in the methods used to analyze semen, as well as the statistical methodology used for the meta-analysis.

Reanalyzing the data of the studies included in the meta-analysis of Carlsen and coworkers (1992), Becker and Berhane (1997) found that the decline in sperm count was significant in the USA alone, the only country included in the meta-analysis with data available over 50 years. In a more extensive reanalysis of the data of Carlsen and colleagues (1992), Swan et al. (1997) confirmed a mean significant decline in sperm concentration of 1.5% per year in the USA between 1938 and 1988 and found a mean significant decline of 3.5% per year in Europe between 1971 and 1990. A new analysis of an expanded set of studies by Swan and colleagues (2000) confirms the decline in semen quality and suggests that the trends in semen quality cannot be attributed solely to confounding factors.

The above reports and commentaries have stimulated various laboratories to analyze their own data. Several publications have reported the results of longitudinal retrospective analyses of semen characteristics of more or less homogeneous groups of men recruited in a single center for a long period of time (Table 5.1). The studies noted in Table 5.1 should not be given equal weight, because they vary considerably in experimental design and sample size. It should also be noted that the time spans covered are not as wide and do not go as far back as does the meta-analysis of Carlsen et al. (1992), the majority commencing in the 1970s or 1980s. The

Table 5.1 - Summary of Longitudinal Retrospective Studies of the Sperm Count Made in a Single Center

		Period	Number	Main\of the Men	Concentration	of Sperm
1	Paris (France)	1973–92	1,351	Unselected potential donors for AI, fathers	↓	↓
2	Ghent (Belgium)	1977–95	416	Unselected potential donors for AI, unknown	↓	=
3	Turku (Finland)	1967–94	5,481	Infertile couples	=	=
4	Edinburgh (UK)	1984–95	577	Research donors, fathers and unknown		↓
5	Toulouse (France)	1977–92	302	Unselected potential donors for AI, fathers	=	=
6	Minnesota (USA)	1970–94	662	Prevasectomy, fathers and unknown	↑	NA
6	New York (USA)	1972–94	400	Prevasectomy, fathers and unknown	↑	NA
6	LA (USA)	1978–94	221	Prevasectomy, unknown	=	NA
7	Seattle (USA)	1972–93	510	Research donors	↑	↑
8	Athens* (Greece)	1977–93	2,385	Infertile couples	↓	NA
9	France**	1989–94	7,714	Partners of women with tubal disease	↓	NA
10	Pisa (Italy)	1970–90	4,518	Infertile couples	↓	NA
11	Sydney (Australia)	1980–95	509	Unselected potential donors for AI, fathers	=	NA
12	Odense (Denmark)	1990–96	1,055	Partners of women with tubal disease	=	NA
13	Münster (Germany)	1977–93	187	Research donors	=	=
14	Jerusalem (Israel)	1980–95	188	Unselected potential donors for AI, unknown	=	NA
15	Southern Sweden	1985–95	718	Infertile	↑	=
16	Canada	1984–96	48,968	Infertile couples	=	NA
17	Copenhagen (Denmark)	1977–95	1,927	Unselected potential donors for AI, unknown	↑	↑
18	Barcelona (Spain)	1960–96	22,759	Infertile	=	NA
19	Slovenia	1983–96	2,343	Partners of women with tubal disease	=	=
20	Magdeburg (Germany)	1974–94	5,149	Infertile couples	↓	NA
21	Hamburg (Germany)	1956–80	36,000	Infertile couples	↓	↓
22	Berlin and Leipzig (Germany)	1985–96	3,821	Infertile couples	↓	↓

*Results from three different laboratories in Athens. **Results from 77 centers. Abbreviations: AI, artificial insemination; unknown, unknown fertility; ↓, significant decline; ↑, significant increase; =, no significant change; NA, not available. References: **1**, Auger et al., 1995; **2**, Van Waeleghem et al., 1996; **3**, Vierula et al., 1996; **4**, Irvine et al., 1996; **5**, Bujan et al., 1996; **6**, Fisch et al., 1996; **7**, Paulsen et al., 1996; **8**, Adamopoulos et al., 1996; **9**, De Mouzon et al., 1996; **10**, Menchini Fabris et al., 1996; **11**, Handelsman, 1997; **12**, Rasmussen et al., 1997; **13**, Lemcke et al., 1997; **14**, Benshushan et al., 1997; **15**, Berling and Wolner-Hanssen, 1997; **16**, YoungLai et al., 1998; **17**, Gyllenborg et al., 1999; **18**, Andolz et al., 1999; **19**, Zorn et al., 1999; **20**, Glöckner et al., 1998; **21**, Licht, 1998; **22**, Thierfelder et al., 1999.

origin of the samples is also an important consideration. For example, men requesting vasectomy are not comparable to men not requesting such surgery. Similarly, studies including men from infertile couples should be viewed with caution because criteria for infertility have changed over time, the proportion of couples seeking assistance is now much greater, and treatment options have changed radically in recent years.

Nine studies included infertile men (Vierula et al., 1996; Adamopoulos et al., 1996; Menchini Fabris et al., 1996; Berling and Wolner-Hanssen, 1997; YoungLai et al., 1998; Glöckner et al., 1998; Licht, 1998; Thierfelder et al., 1999; Andolz et al., 1999). All but one included more than 1,000 men (Table 5.1). Declines in sperm count were found in Athens, Pisa, France, and Germany, an increase was found in southern Sweden, no change was observed in Turku and Barcelona, and no change, an increase, or a decrease was found at different centers in Canada. It is impossible to draw any conclusions from these studies because the availability of infertility services and the behavior of couples wanting to become parents have changed tremendously during the last 30 years in the European countries where most of these studies were done. For example, a laboratory becoming more specialized in male infertility would increase the proportion of severely infertile men recruited, resulting in a decrease in mean sperm count.

Six studies included unselected potential donors for artificial insemination. In three of them (Paris, Toulouse, and Sydney), all the men were fathers (Auger et al., 1995; Bujan et al., 1996; Handelsman, 1997); in the other three studies (Ghent, Copenhagen, and Jerusalem) they were mainly young students of unproven fertility (Van Waeleghem et al., 1996; Gyllenborg et al., 1999; Benshushan et al., 1997). All these studies were apparently done with standardized methodologies, considering only the first ejaculate and using suitable statistical methods. However, in four of them (Ghent, Toulouse, Sydney, and Jerusalem), the sample size was small; the mean number of men included was less than 35 per year. In two cities (Paris and Ghent), a significant decline in sperm count was found; in three others, there was no change (Toulouse, Sydney, and Jerusalem), and in Copenhagen a significant increase in mean sperm concentration and total sperm count was found. A decrease in the total sperm count was observed only in Paris. Despite rigorous methodologies, sample size limitations preclude firm conclusions and only suggest that there may be geographical differences in semen quality.

Three studies analyzed the temporal trend in semen characteristics of donors recruited for research purposes. A decline in mean and total sperm count was found in Edinburgh (Irvine et al., 1996), but an increase in the same characteristics was found in Seattle (Paulsen et al., 1996). The latter study included men with only one ejaculate and men with multiple ejaculates where mean semen characteristics were calculated. In a third study in Münster, no change was observed (Lemcke et al., 1997).

One study analyzed cryopreserved semen taken from men prior to vasectomy in three U.S. cities (Fisch et al., 1996). An increase in sperm concentration was found in Minnesota and NYC; no change was found in LA. In addition, very different mean sperm counts were found (131.5×10^6/ml in NYC, 100.8×10^6/ml in Minnesota, and 72.7×10^6 in LA), but these differences were not analyzed statistically (Fisch et al., 1996). Moreover, in these three cases the data were collected from very small samples (<20 per year in NYC and LA) for which the fertility status was heterogeneous and often unknown. Additionally, the methodology used to count the sperm differed over the time frame of the study (17–25 years), and the reasons for requesting vasectomy may also have changed over time. Given these limitations, it is not possible to conclude whether there are any real differences between the sperm counts found in these three cities.

Most of the retrospective studies report results from a single center. It is of interest to compare the results obtained from different centers located in the same geographic area, that include healthy men studied at the same time period or at different times. Data from three studies in NYC offer the opportunity to compare sperm concentrations over time (MacLeod and Gold, 1951; Rehan et al., 1975; Sobrero and Rehan, 1975; Fisch et al., 1996) (Table 5.2). MacLeod and Gold (1951), using sperm collected between 1945 and 1951 from partners of pregnant women, reported a mean arithmetic sperm concentration of 107×10^6/ml, with 7% of the men having sperm concentrations below 20×10^6/ml. Rehan et al. (1975) in a study of men who had fathered at least two children and were requesting a vasectomy between 1969 and 1974 found a decline in mean arithmetic sperm concentration to 79×10^6/ml, with 5% of the men having sperm concentrations below 20×10^6/ml. Fisch et al. (1996), in a study of prevasectomy patients between 1972 and 1994, found a mean arithmetic sperm concentration of 131.5×10^6/ml. Several methodological differences may account for the discrepant results. First, the earlier two studies analyzed only the first ejaculate of the men, whereas Fisch et al. averaged multiple ejaculates for an unknown proportion of the men they studied. Thus, a bias toward inclusion of men with higher sperm concentrations may have been introduced (Auger and Jouannet, 1997). Second, because Rehan et al. did not control for fertility potential at the time of semen sampling, they performed a subanalysis of 100 men whose partner was currently pregnant and found similar results. Finally, no study accounted for differences in sperm counting methodology.

The studies analyzed by James (1980), Carlsen et al. (1992), and Swan et al. (2000) and other more recent studies illustrate the very large differences in mean sperm concentration reported in populations of healthy and/or fertile men in various parts of the world (Jouannet et al., 2001). It varies from 131.5×10^6/ml in NYC (Fisch et al., 1996) to 48.5×10^6/ml in Iowa City (Nelson and Bunge, 1974), 52.7×10^6/ml in Thailand (Aribarg et al., 1986), and 54.7×10^6/ml in Nigeria (Osegbe et al., 1986). These

Table 5.2 - Sperm Concentration of Healthy Men in NYC (USA)

Reference	Period under Study	Number of Men	Main Characteristics	Mean Sperm Concentration ($\times 10^6$/ml)	
				Arithmetic	Geometric
MacLeod and Gold, 1951	1945–51	1,000	Partners of pregnant women	107	90
Rehan et al., 1975	1969–74	1,300	Prevasectomy, fathers	79	65
Sobrero and Rehan, 1975	1969–74	100	Partners of pregnant women	82	68
Fisch et al., 1996	1972–94	400	Prevasectomy, fathers and unknown fertility	131.5	NA

NA, not available.

differences suggest a possible geographical diversity in human sperm production. Understanding this diversity could help to clarify the influence of environmental or occupational factors on testis function.

In a study analyzing the semen characteristics of men who attended 11 university centers in Canada from 1984 to 1996, YoungLai et al. (1998) found a significant difference in the mean sperm concentration between centers ranging from 48.6 to 104.5 $\times$ 10^6/ml. The mean sperm count increased over the time frame examined in some centers, decreased in others, while still others remained unchanged. Hence, this study not only supports the hypothesis that there are regional differences but also indicates that factors influencing semen quality may be exerting widely divergent effects in different regions.

In 4,710 unselected fertile potential semen donors in eight different centers for the study and preservation of human eggs and sperm (CECOS) in France, it has been shown that the mean sperm concentration (n) and the total sperm count (N) were significantly different (Auger and Jouannet, 1997). The highest values were found at Lille in northern France (n = 103 $\times$ 10^6/ml; N = 398 $\times$ 10^6), and the lowest values at Toulouse in southern France (n = 86 $\times$ 10^6/ml; N = 259 $\times$ 10^6). All the centers were in university hospitals and employed similar criteria for donor recruitment and semen analysis methods. After adjustment for age and period of abstinence before semen collection, the differences between the eight centers remained statistically significant.

The first study specially designed to measure geographical differences in semen quality has recently been completed in Europe, comparing semen characteristics of homogeneous populations of partners of pregnant women (Jørgensen et al., 2001). This cross-sectional study showed significant differences in mean sperm concentration between fertile men recruited from Copenhagen (77 $\pm$ 66 $\times$ 10^6/ml), Edinburgh (92 $\pm$ 63 $\times$ 10^6/ml), Paris (94 $\pm$ 72 $\times$ 10^6/ml), and Turku (105 $\pm$ 73 $\times$ 10^6/ml). The same differences were observed in mean total sperm count from 276 $\pm$ 240 $\times$ 10^6 in Copenhagen to 412 $\pm$ 312 $\times$ 10^6 in Turku. Interestingly, the differences were still significant after adjustment for age, abstinence delay, and season of study. Significant differences have also been reported between various districts of the same city such as London (Ginsburg et al., 1994) or of the same region such as the Paris area (Auger and Jouannet, 1997).

Viewed as a whole, several of the published reports support the hypothesis that there are time-related decreases in semen quality at least within some regions, as reflected in sperm concentration and, where measured, sperm motility and morphology but do no support the hypothesis that the decline is worldwide. Significant differences between regions in semen quality have been demonstrated. However, the biological significance of these observations and their potential causes remain to be determined. Any relationship with endocrine disruption, although plausible, remains entirely speculative at this time.

5.1.2.3 Factors that may influence human sperm quality and bias interpretation of studies on trends. As for most semen characteristics, sperm concentration varies greatly between and within individuals. There is a 10-fold difference between the 10th percentile and the 90th percentile of the distribution in fertile men (Jouannet et al., 1981). The causes and the consequences of this variability on fertility have been well discussed in recent publications (Mees et al., 1997; Auger and Jouannet, 1997; Jegou et al., 1999; Jouannet et al., 2001; Weber et al., 2002). Factors that may influence sperm count and that may explain part of the variability can be classified into three categories: those linked to the characteristics of the men included in the studies, those depending on the methodologies used to analyze the semen or to analyze the data, and those depending on external factors, influencing testis function. Factors relating to the first two categories are listed in Table 5.3. Either information on these factors was not available in most of the reported retrospective studies, or they were poorly considered during the data analysis. The definitions of the populations were particularly vague.

Genetic factors cannot explain the rapid temporal trends that have been described but could explain geographical variations. For example, it is known that Y deletions may be responsible for major spermatogenesis defects (Ma et al., 1993; Reijo et al., 1995). Similarly, differences in gene composition or activity may explain, at least in part, the variability of sperm production within healthy men. Polymorphisms in genes of the Y chromosome have been shown to influence sperm concentration in healthy, fertile Japanese men classified according to their Y haplotypes (Kuroki et al., 1999), and the proportions of men with particular haplotypes varies with ethnic origin (Shinka et al., 1999). It has been demonstrated that fertile brothers of infertile men have lower sperm counts and quality than other fertile men (Czyglik et al., 1986). This observation, which was the first to identify a clear familial component to normal human spermatogenesis, has since been confirmed by Auger et al. (1995). In a study of 17 twin pairs (11 monozygotics and 6 dizygotics), sperm concentration, total number of sperm, and testis volume showed strong familial congruence (Handelsman, 1997). Although these sibling studies do not distinguish between genetic or shared environmental influence, they suggest that a familial factor should be taken into account as a source of variability in the comparison of sperm quality. The very low mean sperm count of fertile men from Africa or South East Asia (Osegbe et al., 1986; Aribarg et al., 1986; Chia et al., 1998) also suggests that an ethnic factor could influence spermatogenesis. The available data, however, do not permit determination of the relative contribution of the respective influences of ethnic, environmental, or other factors, such as sexually transmitted disease incidence, to the reported differences.

In order to draw valid conclusions from comparison of semen values between various studies or within a single study, the sample size analyzed and the role of interindividual variability in sperm production should be delineated. Very large groups of men are required to measure temporal or geographical variations (Berman et al., 1996). In the meta-analysis published by Carlsen et al. (1992), 12 studies included fewer than 20 men and 29 included fewer than 50 men. In 8 of the 10 longitudinal retrospective studies on healthy men published after 1994, the mean number of men included per year was less than 50 (Table 5.1). In such a situation, small changes

Table 5.3 - Factors That May Influence Semen Characteristics Apart from Environmental Chemicals

Population Characteristics	Methodology
Origin of the men	Mode of semen collection
Occupation	Semen analysis methods
Age	Number of ejaculate
Previous medications and diseases	Sexual abstinence delay
Dietary, clothing, smoking habits	Intra- and intertechnician variability
Stress	Season
Fertility	Statistical methods
Sexual activity	
Excessive heat exposure	

in the study or sample characteristics may have an important influence on the temporal trend (Auger and Jouannet, 1997). It is also important to limit the influence of thd variability between technicians and/or laboratory on semen analysis results. A quality assessment made by the four trained European groups who participated in the cross-sectional study cited earlier (Jørgensen et al., 2001) calculated that if the number of samples is 100, a difference of 26% in the geometric medians of sperm concentration will result in a statistically significant difference at the 5% level. If the number of samples is 500, a difference of 18% will be statistically significant (Jørgensen et al., 1997). The size of the populations, their homogeneity, and/or standardization of several factors are important in order to compare data between locations or from one time to another (Table 5.3).

None of the published studies is representative of the general population (Handelsman, 1997). Self-referred volunteers are often better educated people or men with doubts about their own fertility and consequently eager to participate to get information on their own health status. Although it is impossible to make a valid evaluation in the general population, it would be valuable to determine if the same trends exist in various well-defined categories of men, such as young military conscripts, fertile partners of pregnant women, unselected sperm donors, men cryopreserving sperm before a vasectomy, or unselected infertile men.

Although the methodological parameters listed in Table 5.3 are not always taken into account in most studies, they need to be carefully addressed. For example, because of the large intraindividual variability in sperm concentration, comparisons based on the means of several timed ejaculates from each subject would be more appropriate. This, however, may select the men with the highest sperm counts (Auger et al., 1995).

Control for confounding factors, such as age, sexual abstinence delay, or seasons, could be introduced in the statistical analysis to enable better comparisons of data from various centers or times. This has been done in several studies presented in Table 5.1, although the details given are rarely sufficient to permit calculation of adjusted values, which would allow better comparisons of the data. In the study comparing the semen quality in eight different centers in France (Auger and Jouannet, 1997), the adjustment of data to account for men's age and duration of sexual abstinence before semen collection clearly lowered the range of variations of mean and median characteristics between centers. These factors should be included in multiple regression models to compare data of various populations and to measure the effect of external factors.

5.1.2.4 Evidence from hormone and chemical exposures in humans. The Sharpe and Skakkebaek (1993) hypothesis would predict that exposure to hormonally active drugs during gestation should have an effect on later sperm quality, provided free hormone reached the embryo and fetus. The consequences of *in utero* exposure to DES on male reproductive function has been reviewed by Swan (2000). Although sons of DES mothers have an increased incidence of reproductive tract abnormalities, the results for semen quality are conflicting because some studies found reductions in semen quality and others did not. Fertility does not appear to be affected (Wilcox et al., 1995). The fertility of offspring, born between 1958 and 1963, with *in utero* exposure to pharmaceutical estrogens and progestins other than DES, was examined in a retrospective study of 1,888 men and women and 2,044 age-matched controls in Finland (Hemminki et al., 1999). No significant impact of *in utero* exposure to estrogens and progestins on the fertility of offspring could be demonstrated in this study, except that fewer exposed than unexposed men had their first live-born child within one year after getting married. The semen quality of the men was not analyzed.

In an analysis of the semen of men attending an infertility clinic in Calgary, Canada, in 1990–1991, a lower sperm concentration and motility and an increased rate of abnormal sperm (tapered head) were found among 55 agricultural workers. However, the difference in sperm concentration was not statistically significant when compared with the influence of other occupational factors; a more significant dose–response relationship was found between the level of perceived job stress and various abnormalities of sperm morphology and motility (Bigelow et al., 1998). Moreover, this is a difficult study to interpret since attendees at infertility clinics all have the potential for abnormal sperm. In another study, although a significant decline in median sperm concentration was found in Danish agricultural workers after the spraying pesticide season compared with before the season, an equal decline was found in the control group where men were not spraying pesticides (Larsen et al., 1999).

Two studies have been done to analyze the relation between semen characteristics and the concentration of environmental chemicals in seminal plasma. Dougherty et al. (1980) found a negative correlation between the sperm count and PCBs and other organochlorine compounds in the semen of 132 healthy students. A study in 170 men with variable fertility status found that the concentration of PCB congeners was inversely correlated with sperm motility in samples with a sperm concentration of $<20 \times 10^6$/ml (Bush et al., 1986).

5.1.2.5 Conclusions on sperm quality and testis function. Although an extensive review of the published data suggests that there could be temporal and geographical variations in human sperm production, it is not possible to conclude that the phenomenon is real and, if so, to what extent reductions in sperm count may affect fertility. The data should be interpreted with caution. To date, most of the published studies have been retrospective and report data from men recruited for other purposes (donation for infertile couples or research, prevasectomy cryopreservation, infertility diagnosis, etc.) rather than for the analysis of temporal or geographical variations. Recruitment could be very different in the various centers. Additionally, many biases, depending on the methods of analysis employed or the characteristics of the men, could influence the results. These, however, were not taken into account in most studies. Cross-sectional studies of well-defined populations are already underway in several countries but tend to have poor participation rates (Jørgensen et al., 2001).

As discussed above, biologic plausibility and experimental evidence support the hypothesis that chemical agents acting as EDCs could induce testis dysfunction. Moreover, the increasing trends seen in the incidence of testicular cancer (section 5.4.4) and possibly male reproductive tract abnormalities (section 5.1.6) (outcomes that share similar developmental origins and influences) increase the biological plausibility of an EDC influence on a constellation of effects, including sperm quality. However, the lack of any clear effect of the pharmaceutical estrogen DES on human testis function and the lack of any demonstration to date of an endocrine-disrupting mechanism for other chemical exposures indicate the need for more studies before firm conclusions can be drawn. Outcomes in relation to exposure to EDCs need to be analyzed at two different times – exposure before birth or perinatally, when EDCs could alter the testis and genital tract development, and exposure after puberty, when a toxic effect could impair spermatogenesis.

5.1.3 Fecundity and Fertility

5.1.3.1 Methodologies showing temporal and geographical trends. Fecundity, fertility, and "sperm quality" are distinct parameters that are not equivalent and are frequently confused. Sperm count and sperm quality do not necessarily predict whether conception will take place for a given couple. A "fertile couple" has conceived at least one child. Fecundity is the ability of a couple to conceive a child and is often evaluated by the time necessary to achieve pregnancy. "Time to pregnancy" is a useful epidemiological tool to measure the fecundity of a population. It does not require categorization of subjects into fertile and infertile groups—a method with significant limitations. The model has been validated as a consistently effective tool for measuring the impact of exogenous agents that affect reproduction. For example, studies have shown that there is a clear difference in the time to pregnancy among nonsmokers and smokers (e.g., van der Pal-de-Bruin et al., 1997).

Because of the complexity of human reproduction, it is often difficult to determine whether or not there is an actual increase in age-specific infertility rates. Published studies on temporal or geographical trends in human fecundity are sparse. However, the time to pregnancy has been observed to be shorter among couples in Finland than in the United Kingdom (Joffe, 1996), a finding that fits with the view of most published studies that sperm counts are higher in Finland (Suominen and Vierula, 1993; Vierula et al., 1996; Jensen et al., 2001). By comparison, although semen quality has been reported to have declined in the United Kingdom (Irvine et al., 1996), a retrospective study of the British population 16–59 years old on time to pregnancy for all births conceived after unprotected intercourse that began during 1961–1993 has revealed an increase in fertility rather than a decline (Joffe, 2000). Because parity and body mass index have been used as indicators of maternal estrogen levels, and being the first child and being the son of a moderately obese mother are known risk factors for cryptorchidism and testicular cancer, it was reasoned that these factors may also be associated with decreased fecundability (Joffe and Barnes, 2000). In a cohort study using a representative sample of the Brhtish population born in 1958 that have been followed since birth, the effect of the age of the parents of subjects at the time of their birth, as well as numerous other characteristics (smoking, body mass index, height, parity of mothers, and social class), on time to pregnancy was evaluated. A total of 1,714 men and 2,587 women provided data on time to pregnancy. All of the ORs were in the vicinity of 0.9–1.1. Even *in utero* exposure to cigarette smoke was not associated with any effect on female time to pregnancy as an adult, in contrast to previously published reports. These data therefore suggest that the heterogeneity in fertility is not related to any of the factors examined in this study. Thus, no clear picture emerges with respect to time trends and human fertility.

Differences in time to pregnancy have been found in a prospective study involving seven well-defined geographical areas in Europe (Juul et al., 1999). The highest fecundity was observed in southern Italy and northern Sweden; the lowest fecundity was in east Germany. The differences in time to pregnancy remained significant after adjustment for regional differences in body mass, smoking, frequency of intercourse, and sexually transmitted disease. The longest time to pregnancy was observed in Paris, and the shortest in Rome; however, no sperm data are available from the same European regions to allow association of the observed difference in time to pregnancy with a male factor. A subsequent European study has compared time to pregnancy and sperm quality in fertile couples from Copenhagen (Denmark), Paris (France), Edinburgh (Scotland), and Turku (Finland) (Jensen et al., 2001). After adjustment for confounders, this confirmed a decreased probability of conception in Paris couples compared with couples from the other three cities, where there were no differences in time to pregnancy. The difference was not due to any difference between regions in sperm quality. The authors noted the low participation rates could have resulted in selection bias.

In Sweden, analysis of birth registries has shown that the population of subfertile women (defined as those who did not become pregnant after more than 1 year) has actually decreased from 12.7% in 1983 to 8.3% in 1993 in the general population (Akre et al., 1999a). The decrease, which was independent of maternal age, could not be associated with any trend in semen quality but was probably linked to a decrease of sexually transmitted disease incidence in Sweden.

Another potentially useful approach is to review the total fecundity of a population of people with no predisposition for limitation of family size. For example, there has been a declining age-specific fertility rate in the Hutterite population, a group in which reproductive practices are unlikely to have changed over time (Nonaka et al., 1994; Sato et al., 1994). These retrospective cohort studies revealed a decline in the total number of children born beginning with a cohort 1931–1935 and a continuing decline with subsequent birth cohorts. Although genetic influences cannot be excluded, neither can extraneous factors such as man-made chemicals, and covert birth control practices, although there is no clear link to an endocrine disruptor mechanism.

5.1.3.2 Evidence from chemical exposures in humans. Occupational exposures are often cited as evidence of external impacts on fertility (Schaumburg and Molsted, 1989; Sitarek and Berlinska, 1997; Feinberg and Kelly, 1998). The literature on chemical and other occupational exposures is extensive. Only the studies potentially relevant to EDCs are reviewed here. Published studies relate to the impact of both female and male occupations. In one case–control study, in which 281 women with a diagnosis of infertility were compared with 216 postpartum women, women with a history of working in the agricultural industry had an elevated risk of infertility (Fuortes et al., 1997). Several epidemiological studies have been undertaken to study the fecundity and fertility of farmers exposed to pesticides. A retrospective study of 43 couples in The Netherlands whose respective male partner was a fruit grower included 91 pregnancies from 1978 to 1990 (de Cock et al., 1995). Exposure to pesticides was determined by self-reported data. An adverse effect of pesticide exposure was found, mainly apparent in highly exposed men who tried to conceive during the spraying season. The incidence of couples consulting a physician because of a fertility problem was also much greater in the high-exposure group; however, there is no clear link to an endocrine disruption mechanism. In a follow-up on-going case–control study on occupational exposures and semen quality among couples consulting an infertility clinic, among 899 men who delivered a semen sample, an association between impaired semen parameters and aromatic solvent exposure was observed, but no association was found with pesticide exposure (Tielemans et al., 1999a). However, significantly decreased fertilization rates were observed for couples with male partners exposed to pesticides and enrolled in an *in vitro* fertilization program (Tielemans et al., 1999b). Adjustment for paternal or maternal smoking habits, caffeine use, alcohol consumption, or other occupational exposures had little effect on the observed

association. In a retrospective study of 2,012 farm couples, no strong or consistent pattern of association of exposure to various classes of pesticides with time to pregnancy could be observed (Curtis et al., 1999). Similarly a large study in Denmark and France on exposure to pesticides and a control group of agricultural workers did not demonstrate any effect of pesticide exposure on time to pregnancy (Thonneau et al., 1999). Although no association with endocrine disruption has been demonstrated in any of the foregoing studies, it can be concluded that human fecundity and fertility are reduced in some occupational groups with exposure to man-made chemicals and that there is a need to undertake mechanistic studies to better characterize the association.

In addition to occupationally exposed individuals, at-risk groups may also include those whose social or leisure activities lead to higher exposures than occur in the general public. Changes in fecundability were investigated in the New York State Angler Cohort Study and lifetime exposure to PCBs was estimated from recent consumption of contaminated Great Lakes fish (Buck et al., 1999). Maternal consumption of fish for 3–6 years was associated with reduced fecundability (OR, 0.75; 95% CI, 0.59–0.91). However this effect was no longer significant in those with more than 7 years of fish consumption (OR, 0.75; 95% CI, 0.51–1.07). Maternal consumption of more than one fish meal a month was also associated with reduced fecundability (OR, 0.73; 95% CI, 0.54–0.98), whereas there was no association with paternal fish consumption or with either maternal or paternal estimated lifetime exposure to PCBs. These data suggest that maternal consumption of contaminated fish may reduce fecundability but are insufficient to draw any conclusions about paternal consumption.

Results differing from those above have been reported by others on the relationship between time to pregnancy and the consumption of sport fish containing PCBs and mercury (Courval et al., 1999). In this study, lifetime exposure to PCBs and mercury was estimated by the numbers of fish meals consumed. An increased time to pregnancy was observed with increased paternal consumption (adjusted ORs were 1.4, 1.8, and 2.8 for 1–114, 115–270, and 271–1,127 annual fish meals consumed, respectively). Only in the highest fish consumption group did the 95% CI for the OR exclude 1.0. No relationship was found for women consuming sport-caught fish. These data suggest a weak association only between high fish consumption in men and conception delay.

The studies have obvious weaknesses in that they assume a constant pattern of consumption, that levels of contamination in fish remained constant over time, that the same types of fish were consumed throughout, and that the individuals' susceptibility to adverse effects was constant over the periods under investigation. Nevertheless, these studies indicate that anglers may be an at-risk population for the effects of EDCs from fish.

The possible influence of dietary phytoestrogens should also be considered. It has long been known that grazing on clover-rich pastures high in phytoestrogen precursors causes infertility in sheep (Bennetts et al., 1946). More recently, studies have shown prolongation of the menstrual cycle in healthy premenopausal women given soy protein daily containing 45 mg of isoflavones, attributed to prolongation of the follicular phase due to supression of the normal midcycle surge in FSH and LH (Cassidy et al., 1994, 1995; Lu et al., 1996), although in another study on flaxseed ingestion it was the luteal phase that was reported to be prolonged (Phipps et al., 1993).

Actual chemical exposure data in relation to fertility are limited. One example of what might be possible using new reproductive technology techniques is the isolation of persistent organochlorine chemicals from ovarian follicular fluid of women undergoing *in vitro* fertilization (Jarrell et al., 1993a, 1993b). Isolation of such chemicals at a critical period of oocyte development may provide an important biomarker of exposure and potential outcome.

5.1.3.3 Evidence from animal studies. There are numerous animal study protocols designed to identify the potential hazards of chemical exposure to fertility. One of these, RACB has been used extensively, although it should be noted that RACB was not designed specifically to detect EDCs. The data for 72 chemicals contained in the RACB database were evaluated (Chapin et al., 1997b) to identify which of the definitive measures (sperm counts and necropsy results) were the most predictive of apical concerns (fertility). Longer estrous cycles in mice were correlated with reduced numbers of pups, a relationship that was stronger in F_1 than in F_0 generation animals and not seen in the controls. Fertility was reduced if >15% of the sperm had abnormalities or sperm motility was <37%. These estimates were not improved by including sperm count in the model. The data suggest that epididymal sperm counts, motility, estrous cycle length, and testis and epididymal weights are useful although not complete surrogates for overall reproductive function. Epididymal sperm counts correlate well with fertility over numerous studies such that a reduction of 20% results in reduced fertility. Although these studies point to target organs affected by the test compound, they do not provide evidence that endocrine disruption is involved as the primary mechanism mediating the adverse effect.

5.1.3.4 Conclusions on fecundity and fertility. The foregoing studies demonstrate an association between delayed conception and exposure to high levels of environmental contaminants. However, the relationship between changes in time to pregnancy and endocrine disruption remains speculative, due to the complex array of issues that may alter normal human reproduction and result in a longer time to pregnancy. Moreover, time to pregnancy is dependent on the characteristics of both partners (Weinberg et al., 1994). The time to pregnancy model could, however, be useful in providing corroborative evidence in exploring the significance of geographical differences in sperm quality.

5.1.4 Spontaneous Abortion

5.1.4.1 Methodology and pathobiology. The definition of spontaneous abortion is a pregnancy lost prior to viability, typically defined as 20 weeks from the first day of the last menstrual period, of a fetus weighing less than 500 g. The incidence of spontaneous abortion is estimated to be 50% of all pregnancies, based on the assumption that many pregnancies abort spontaneously with no clinical recognition. The difficulties associated with the measurement of early fetal loss have been discussed elsewhere (Lasley and Overstreet, 1998).

The known causes of spontaneous abortion are primarily chromosomal abnormalities in the first trimester (French and Bierman, 1972). In the second trimester, spontaneous abortions are often attributed to uterine abnormalities. Risk factors for spontaneous abortion include advanced maternal age, increasing parity, increasing paternal age, previous spontaneous abortions, therapeutic agents such as chemotherapy, and radiation and anesthetic agent exposures. Tobacco and ethanol exposure also have an impact, alone and in combination, and substance abuse related to cocaine and other drugs may also be associated with fetotoxic responses (Arbuckle and Sever, 1999).

The effects of environmental influences on spontaneous abortion are difficult to explore. A recent review (Arbuckle et al., 1999) has

illustrated the problems of investigating any associations between pesticide exposures and spontaneous abortion and showed that there is a substantial need to improve methodology. Much of the critique is equally applicable to other chemical exposures. There are data gaps regarding the nature of the exposure of women, confirmation of such exposure by chemical analysis in the blood, sample sizes, selection biases from low participation rates, poor differential recall rates among women with spontaneous abortions and control subjects, case ascertainment rates based on time of pregnancy diagnosis, poor confirmation of the existence of pregnancy, and poor control for previous spontaneous abortion. Furthermore, many of the pesticides have not been studied for their relationships to abortion. In most of the studies to date, no measured exposure concentrations are available to correlate with effects (Arbuckle et al., 1999).

5.1.4.2 Evidence from chemical exposures in humans. Of all pesticide exposures, phenoxyherbicides have been studied most. Ashengrau and Monson (1989, 1990) have shown that paternal exposure to 2,4-D increased the risk of abortion. Although DBCP has been principally related to male infertility, it has also been associated with spontaneous abortion (Potashnik et al., 1984). Wives of workers exposed to organochlorine pesticides have shown an elevated risk of spontaneous abortion and stillbirth (Rupa et al., 1991). Levels of DDT were found to be higher among women with abortions or stillbirths in India (Bercovici et al., 1983; Saxena et al., 1983). There is also evidence that organochlorine and carbamate pesticides cross the placenta and possibly cause fetal death (Arbuckle and Sever, 1999).

Savitz et al. (1997) analyzed the outcome of 3,984 pregnancies in 1,898 couples of farmers in Ontario included in the 1986 Canadian Agriculture Census. The man's exposure to pesticide was based on his experience in a 3-month window of time before conception. An increased spontaneous abortion rate associated with reported use of thiocarbamates, carbaryl, and unclassified pesticides was observed. There was also an association between the use of triazines, particularly atrazine, and 2,4-D and an increased risk of preterm delivery.

Women exposed to HCB as children, who developed severe porphyria cutanea tarda, have been followed for approximately 40 years. HCB residues are still present in many of the survivors. During a review of the reproductive outcomes of these women, an unexpected finding was the association of high serum concentrations of HCB levels with high rates of spontaneous abortion. This finding was evaluated in a population-based methodology to include two control populations. The effect was present when all observations were included, indicating the effect was measurable down to concentrations at detection limits (Jarrell et al., 1998). These observations need to be corroborated in further studies.

A causal link has not been demonstrated in any of the above examples between pesticide exposure and spontaneous abortion, nor has an endocrine disruptor mechanism been established. However, a number of pesticides have been shown to have estrogenic, antiandrogenic, or antiprogestagenic activity (Chapter 3, section 3.12). In the context of spontaneous abortion, it is compounds acting on progesterone, critical for implantation and the maintenance of human pregnancy (Csapo et al., 1976), which are of most interest. Interference with either progesterone production by removing the corpus luteum (Csapo et al., 1976) or, alternatively, inhibiting progesterone function by administration of an antiprogestin such as mifepristone (RU-486; Scheepers et al., 1999), can result in a spontaneous abortion. It is also well known that high doses of estrogen, the so-called "morning-after pill," can be used to prevent implantation following unprotected intercourse. These observations suggest theoretical mechanisms by which abortions might be induced by environmental chemicals, but dose considerations cast some doubt on the probability that low-level environmental chemical exposures would have an effect, unless the chemicals were accumulated in the body.

5.1.4.3 Evidence from animal studies. In this context, a comparison of the animal and human data on HCB is of interest. Foster et al. (1995) demonstrated that *in vivo* administration of HCB monkeys reduces serum progesterone concentration in the luteal phase of the cycle. The mechanism is not understood, although it may involve a reduction in steroid-metabolizing function in the ovary or adrenal. It is possible that HCB could be functioning as an endocrine disruptor by reducing luteal phase serum progesterone and limiting fetal survival. The persistence of this chemical in fat could explain the effect observed in women over many years (see section 5.1.4.2).

The effect of EDCs on preimplantation loss in rodents has been investigated in a decidual cell response model (Cummings, 1993). In this model, pregnant rats are dosed with the test compound on GD 1–8 and, sacrificed on GD 9, and the number of implantation sites was recorded. Ketoconazole, an imidazole antifungal agent that has been shown to alter mammalian steroidogenesis, was shown to decrease the number of implantation sites (Cummings et al., 1997). The decidual cell response was blocked in parallel with ketoconazole-induced suppression of serum progesterone. Because the effects of ketoconazole were absent in long-term ovariectomized and hormone-replaced rats, these data reveal that the effects of ketoconazole on the decidual cell response are via direct effects on ovarian steroidogenesis that specifically results in decreased serum progesterone levels. These data therefore provide a potential mechanism whereby EDCs may play a role in early pregnancy loss.

5.1.4.4 Conclusions on spontaneous abortion. In summary, there are substantial gaps in our knowledge about whether exposure to environmental chemicals has an impact on spontaneous abortion rates. Epidemiological studies with more rigorous methodology are required, but the difficulties of detecting early fetal losses, which may be of more relevance than later clinically recognized spontaneous abortions, should not be underestimated.

5.1.5 The Sex Ratio

5.1.5.1 Temporal trends in the sex ratio. The sex ratio is defined as the number of male births divided by the number of female births. It has been suggested that the sex ratio is a potential sentinel marker for population health analysis (Davis et al., 1998). An important assumption is that the sex ratio normally remains stable over a long period of time, although this intuitive position may be challenged with the availability of data from longer studies of populations.

Declining sex ratios (fewer males) have been recorded for a number of regions including Canada (Allan et al., 1997), the United States (Allan et al., 1997; Scialli et al., 1997), The Netherlands (Pal de Bruin et al., 1997), and Denmark (Møller, 1996). Additional information shows apparent declines in the sex ratio in Sweden, Germany, Norway, and Finland (Møller, 1996, 1998). Reductions have also been noted in Latin American countries (Feitosa and Krieger, 1992, 1993). In contrast, a trend toward an increase in the sex ratio has been reported for Italy, Greece, and The Netherlands (Astolfi and Zonta, 1999).

The magnitude of the decrease in the sex ratio over the 20-year period from 1970 to 1990 has been estimated in North America,

where there were around 8,000 fewer males in Canada and 38,000 fewer males in the United States over this period of time (Allan et al., 1997; Scialli et al., 1997). It should be noted, however, that large population studies are required to address this issue. The Allan et al. (1997) study determined that 4.7 million live births over 20 years would be required to measure such reductions with an alpha value of 0.05 and a beta value of 0.9. It is important that population studies on trends in the sex ratio provide such power considerations.

There may be racial differences in sex ratios within countries. Regression analysis of temporal trends in sex ratio of live births between 1969 and 1995 in the United States (Marcus et al., 1998) revealed a significant decline among whites for the 27 years under study (OR, 0.9935; 95% CI, 0.9919–0.9952). In contrast, the sex ratio among blacks during the same time period revealed a significant increase in the sex ratio (OR, 1.0208; 95% CI, 1.0162–1.0254).

Concerns have also been raised that the effects are not homogeneous in the population throughout the entire country. In the Allan et al. (1997) study, the reductions were expressed most strongly in the eastern part of Canada, with the lowest ratios detected in the Atlantic Provinces and Quebec. Astolfi and Zonta (1999) examined the trends for birth sex ratios in metropolitan compared with nonmetropolitan areas of Italy. In this study, there was a negative trend in live born males in metropolitan areas that was significantly different from the positive trend observed for nonmetropolitan areas. There are also variations in the distribution noted in the United States, with significant declines present in four of nine regions (east-north central, west-north central, south Atlantic and Pacific; Allan et al., 1997).

5.1.5.2 Evidence from chemical exposures in humans. Although data are limited on factors that change the sex ratio, there is clear evidence that external influences are associated with such a change. These factors can be grouped into medical, occupational, and environmental. The reported medical factors shown or suggested to reduce the sex ratio include older age fathers and mothers, *in vitro* fertilization, ovulation induction, non-Hodgkin's lymphoma, hepatitis, and multiple sclerosis (Ruder, 1985; James, 1976, 1980, 1994, 1995a, 1996, 1997).

A theory regarding the control of events at the time of fertilization proposes that the hormonal status of the parents at the time conception is a key consideration (James, 1986). Clinical observations are consistent with this theory. For example, there have been numerous reports that ovulation induction with clomiphene citrate in women results in fewer male births. These have been most recently summarized in a large meta-analysis (Jarrell et al., 1993a, 1993b). The processes involved in ovulation induction clearly interfere with baseline hormonal homeostasis. The changes observed in sex ratio following ovulation induction could be due to endocrine disruption at either pharmacological or toxicological levels, from direct effects of the drug itself and/or the effects created by the changing endocrine environment. Another relevant observation is the report of a higher sex ratio when men were administered testosterone during attempts to conceive (Sas and Ezollosi, 1980). Although the sample size was small, the findings are consistent with the relative estrogen:androgen balance.

A common mechanism underlying decreasing fertility, testicular cancer, and changes in sex ratio has been proposed (Jacobsen et al., 2000). In this study, the hypothesis that there is an association between testicular cancer, lower fertility, and sex ratio was investigated using the total population of Danish men born between 1945 and 1980. Between 1960 and 1993, 3,530 men developed testicular cancer; 1,488,957 men born in the same time period and their biological children (1,250,989) served as the comparison group. Men who developed testicular cancer had significantly lower fertility rates than did the comparison group and a significantly lower sex ratio (0.957 compared with 1.053). The reduction in fertility was more pronounced in men with nonseminoma tumor type, whereas the reduction in sex ratio was independent of histological tumor type. The authors concluded that the data support speculation that these abnormalities are linked by as yet undetermined biological mechanisms.

Although it is difficult to establish cause-and-effect relationships in the general population, evidence collected from studies of occupational and accidental exposures to man-made chemicals indicate that they may be involved, although whether it is via an endocrine mechanism has yet to be demonstrated. Certain occupational exposures have been significantly associated with changes to the sex ratio. Men exposed to the pesticide DBCP experienced profound semen deficiency and severe disruption in fertility (Goldsmith, 1997), with significant reductions in the numbers of males born to women whose partners were exposed to DBCP (Potashnik et al., 1984). The proportion of male births observed for the same men prior to exposure was 0.5. However, the mechanism by which this chemical would have changed the sex ratio is not completely understood, although damage to Y-chromosome-bearing spermatozoa has been proposed (Goldsmith et al., 1984), perhaps by a non-endocrine-mediated chelating mechanism. Occupational exposure to organochlorines has also been suggested to alter the sex ratio. A Netherlands study of offspring born from 1978 to 1990 revealed a shift toward daughters when men had workplace exposure to pesticides (de Cock et al., 1995). Exposure to the pesticide vinclozolin, an antiandrogen, has also been implicated (Zober et al., 1995). Other occupational exposures reported to be associated with a change in the sex ratio have included working in the aluminum industry as "carbon setters," "anode setters," or "carbon changers" (Milham, 1993) and exposure to waste anesthetic gases (Wyatt and Wilson, 1973). Changes have also been reported to result from exposure to inorganic borates, alcohol, lead, and solvents (Dodds and Armson, 1997). Five retrospective studies of heavily polluted Scottish residential areas also produced evidence of internal exposure altering sex ratio (Williams et al., 1992, 1995). The pollutants involved included emissions from Akron smelters, steel foundries and incinerators in Scotland between 1975 and 1983. However, for none of these occupational or environmental exposures, including the pesticides, is there evidence as yet that the effect is endocrine mediated.

Further evidence that environmental exposures may influence sex ratio comes from an epidemiological report of a population highly exposed to TCDD from a chemical plant explosion in Seveso, Italy (Mocarelli et al., 1996, 2000; Signorini et al., 2000). In 1976, an explosion released a large cloud that dispersed many kilograms of TCDD. Between April 1977 and December 1984, corresponding to one half-life of TCDD, there was a reduction in sex ratio among those with the greatest exposures (48 girls and 26 boys). The effect was greatest among parents with the highest serum levels of TCDD. In fact, none of the nine couples with the highest serum TCDD levels bore a single male. In a recent report (Mocarelli et al., 2000), an increased probability of having a female birth was found with increasing TCDD concentrations in the serum of fathers ($p = 0.008$), starting at concentrations below 20 ng/kg body weight. Moreover, fathers that were exposed to TCDD when they were 19 years old or

younger were significantly more likely to father a girl than a boy (sex ratio, 0.38; 95% CI, 0.30–0.47). Although much larger than the initial study by this team, the sample size is still rather small for a study of this type. The study was conducted with 239 men and 296 women, from which 346 girls and 328 boys were produced between 1977 and 1996. Because 1985 the sex ratio in this population has returned to expected levels (Needham et al., 1997). It should be noted, however, that these findings are based on small numbers and have not as yet been corroborated, and that there were no changes in the sex ratio noted in the TCDD-contaminated region of Kazakhstan (Hooper et al., 1998) or in those with high exposure to PCBs and PCDFs from consumption of contaminated cooking oils in Taiwan (Rogan et al., 1999). All these studies suffer from potential sample selection bias, and the results are based on very small sample sizes, as well. The mechanism for any TCDD-induced change in the sex ratio has not been determined, and although there is an assumption from other work on TCDD that endocrine modifications are involved, this has not been confirmed. In a related report, HCB, which like TCDD binds to the AhR although at a lower affinity, may have been associated with a reduction in the sex ratio (Jarrell et al., 2000).

5.1.5.3 Evidence from animal studies. In animals, several findings suggest that external factors may influence the sex ratio via disruption of the endocrine system. Vandenbergh and Huggett (1994) have shown that in mice the intrauterine position of the mother affected the sex ratio of the offspring. These findings correlated with maternal anogenital distance. The anogenital distance was longer in females located in the uterus between two males (2M) than in females located between no males (0M). The proportion of males in first litters born to 2M females was 58%, for 1M females 51% and for 0M females 42%. This pattern continued into the second litter. There were no changes in the number of pups born to mothers from different intrauterine positions.

Second, there is a recognized difference in developmental rates of male and female embryos. Male embryos in almost every species that has been studied advance to the blastocyst stage before females. This may provide a mechanism for intrauterine discrimination at the time of implantation. Other studies have shown there are differential rates of cell growth and division between XX and XY mammalian embryos (Tsunoda et al., 1985), and the differences are associated with the Y chromosome (Burgoyne, 1993). Differential growth rates have been observed in mice (Burgoyne, 1993), rats (Scott and Holson, 1977), and humans (Pederson, 1980). This more rapid growth of male embryos exists at the preimplantation stage (Pergament et al., 1994).

5.1.5.4 Conclusions on sex ratio. The limited evidence from animal studies suggests that there may be an intrauterine endocrine event that is responsible for altering sex ratio. Other observations on differing growth rates of male and female embryos offer a mechanism whereby the actions of estrogens, antiestrogens, and antiandrogens may be having a gender discordant impact. It is possible, for example, that a drug such as clomiphene citrate, which binds to the uterus and acts as an antiestrogen, may have an effect that is toxic to the more rapidly developing male embryo.

Where the biological plausibility is most challenged, however, lies in the area of how environmental agents might be associated with the temporal trends noted above. Endocrine disruptors present in the environment may indeed have subtle, long-term effects on biological tissues. However, this is quite different from the extreme disruption of the hormonal milieu that occurs during pharmacological ovulation induction. A further note of caution is offered in the recent report of Vartianen et al. (1999). Extending the analysis to 250 years in Finland, it was suggested that changes in sex ratio antedated any exposures to environmental chemicals. This would indicate that recent associations with the emergence of certain broad environmental exposures may be misleading because of too brief a period of analysis. Nevertheless, there is limited evidence for the suggestion that changes in the sex ratio may represent a general trend in society as a consequence of exposure to EDCs. There is also a broader hypothesis that the slight reductions in sex ratio represent a re-equilibration in response to rising sex ratios and improvements in medical care during this century (James, 1995b, 1998). The hypothesis that endocrine-active compounds affect the sex ratio will require further investigation of their action on the mechanisms associated with sex determination, implantation, and embryogenesis.

5.1.6 Male Reproductive Tract Abnormalities

5.1.6.1 Development of the male reproductive tract. In mammalian species, the default phenotypic sex is female. That is, for a phenotypic male, a whole series of events must be triggered and coordinated to develop the male reproductive system and associated secondary sexual characteristics. A failure of certain genes to be expressed or hormones to act normally result in a female phenotype. Much of the understanding of the key effects responsible for normal development comes from a variety of genetic and gene knockout/overexpression studies, where the absence or overexpression of a particular gene has been associated with failure of the male reproductive tract to develop. When the gonad develops from the genital ridge in the embryo, it has the potential to follow either the female or male route. Key to becoming a male is the expression of the sex-determining gene (sry); this in turn elicits a cascade of events that enable the fetal gonad to develop into a testis (Berta et al., 1990). Other genes implicated in normal differentiation of the male reproductive system include, but are not limited to, steroidogenic factor-1 (Ikeda, 1996; Parker et al., 1996), DAX-1 (Guo et al., 1995; Parma et al., 1997), and a variety of homeobox genes (Lindsey and Wilkinson, 1996; Pellegrini et al., 1997).

In the fetal gonad, the development of Sertoli cells plays a major role in how the reproductive system develops. The Sertoli cell synthesizes Müllerian inhibiting substance (Behringer, 1995), which initiates the removal of the female structures (the Müllerian ducts) that would have formed the uterus, oviducts, and so forth. Sertoli cells also control normal development of the Leydig cells, the site of androgen (testosterone) synthesis, which then plays an important role in the development of the epididymis, vas deferens, and seminal vesicles. DHT, the metabolite of testosterone produced via the action of the enzyme 5α-reductase, appears crucial for the development of the prostate and external genitalia. In rodents, most of these events take place in late gestation (for the rat GD 12–20), whereas in the human fetus most events are in the first trimester of pregnancy. Table 5.4 illustrates the various time lines for the development of the male reproductive tract in rats and humans.

5.1.6.2 Ability of experimental protocols to detect chemicals affecting male development. In experimental protocols employed until recently to study developmental toxicity, late gestation (e.g., in rats from GD 16 onward) was not normally included as part of the exposure period. Thus, for chemicals that were not persistent in the dam, potential effects on male reproductive development could have been missed in examination of the fetuses just prior to term. Testing guidelines have now been

Table 5.4 - Time Lines for Male Reproductive Development: Comparison of the Rat with Human

Rat (GD/PND)	Human	Event
GD 8–10	4 weeks	Germ cells migrate to medial aspect of mesopnephros, move to parenchyma, along with precursor Sertoli cells and interstitial cells
~12	5 weeks	Process above is complete
~14	6 weeks	Sex cords start to develop: Sertoli cells identifiable
	7 weeks	Müllerian duct begins to degenerate (influence of Müllerian inhibiting substance)
18	8 weeks	Wolffian duct development begins (testosterone); initiation of androgen secretion
	Late 8 weeks	Seminal vesicles bud off Wolffian ducts
	9 weeks	External genitalia start to masculinize; continues to late gestation (DHT)
	10 weeks	Prostate develops from walls of urogenital sinus (DHT)
19	11 weeks	Serum FSH starts to rise
	13 weeks	Serum LH starts to rise
21	40 weeks	Birth
PND 15	12 years	Sertoli cells stop dividing; the first spermatocytes are present
±22	Last trimester	Testis descent

modified or are being modified to extend exposure to cover the developmental period from implantation to just prior to term.

Multigeneration and other research protocols do have the potential to detect effects of endocrine-active chemicals on male reproductive development because the critical period of exposure is covered. It is only recently, however, that additional end points now known to be particularly sensitive to hormonal status have been or are being incorporated into these testing protocols (US EPA, 1998a, 1998b; US FDA, 1999; OECD, 1999a, 1999b). Most breeding protocols give indications of male fertility, litter size, and sex ratio, but newer end points, in particular, measurements of anogenital distance, retention of thoracic areolae/nipples, and preputial separation have now been included as indicators of normal male reproductive development sensitive to the potential effects of exogenous endocrine-active agents. In normal reproduction studies, the sexing of rat pups is accomplished by observing the distance between the sex papilla and the anus. In males, this distance is approximately twice that of females and is a function of the androgenic status of the animals. Treatments can alter this end point, which can be easily measured, normally on PND 1. The anogenital distance is also a reflection of the size of the pup, and pup body weight should be used as a covariate in the analysis of these data. In the normal male rodent pup, the anlagen of the thoracic nipples regresses under the influence of DHT. Examination of pups before the hair begins to grow (usually around PND 14) and counting the number of areolae and nipples again provide an indication of the hormonal status of the animals. Thus, for example the classical antiandrogen flutamide will result in a female phenotype for male pups with the presence of all thoracic nipples in males (Chapter 3, section 3.12.2.1). Preputial separation is an index of puberty in male rodents when the prepuce separates from the glans penis after androgen-induced apoptosis. It is an end point that is relatively insensitive to body weight changes. Significant delays or advances indicate a change in the androgen status of the animals on test.

5.1.6.3 Evidence from animal studies. There is now considerable evidence from laboratory animal studies of the adverse effects of exposure of males to estrogenic and antiandrogenic chemicals during critical periods of development. Effects induced by exposure to E_2 and other estrogenic chemicals during the period of male reproductive tract development include reduced testis and epididymis weight, reduced sperm numbers and motility, increased prostate weight, and delayed puberty. Principal manifestations of developmental exposure to antiandrogens include reduced anogenital distance, hypospadias, retained nipples, reduced testes and accessory sex gland weights, and decreased sperm production.

Examples of effects in experimental male animals exposed to endocrine disruptors during the prenatal/perinatal period are summarized in Table 5.5. A more detailed discussion of chemicals with known endocrine activity that can affect male reproductive tract development in experimental animals is given in Chapter 3 (section 3.12).

5.1.6.4 Hypospadias and cryptorchidism.

5.1.6.4.1 Known risk factors. Known risk factors associated with failure of the testis to descend into the scrotal sac (cryptorchidism) include ethnicity, a family history of cryptorchidism, use of analgesics during pregnancy (Berkowitz and Lapinski, 1996), birth order (Møller and Skakkabaek, 1996), and maternal obesity (Berkowitz et al., 1996). Several of these are also risk factors for hypospadias, a developmental abnormality in which the urethra opens on the underside of the penis or the perineum (Akre et al., 1999b). Evidence of a seasonal effect with peaks for cryptorchidism occurring at different times of the year in various studies has been reported (Källén et al., 1986; Berkowitz et al., 1996), although the significance of this finding has yet to be determined.

It has been suggested that early exposure to EDCs could cause abnormalities of the genital tract as well as reduce sperm production and induce testis cancer (Sharpe and Skakkebaek, 1993). Cryptorchidism is a well-known risk factor for later development of testis cancer, and in Denmark it has recently been found that subfertile men also have a higher risk of testis cancer (Møller and Skakkebaek, 1996). These observations support the hypothesis that there is a developmental link between certain male reproductive health disorders.

5.1.6.4.2 Temporal trends. There are reports of temporal increases in the frequency of developmental abnormalities of the male reproductive tract, such as hypospadias and cryptorchidism. However, it should be noted that statistics for the birth prevalence of these abnormalities vary widely. For example, Toppari et al. (1995) reported a range for hypospadias from 0.37 to 41 per 10,000 infants and for cryptorchidism from 3 to 1,340 per 10,000 infants. These differences are probably attributable to differing diagnostic and reporting criteria and to ethnic/genetic differences. Such large differences can make comparisons between studies unreliable, and it should be noted that much of the temporal trend data discussed below rely on just such comparisons. Minor forms of hypospadias have generally been estimated to account for around three-quarters of cases, and a rising trend may simply reflect a more frequent or earlier diagnosis of minor forms over time or an increasing tendency to report them to birth defect registries (Dolk, 1998). Thus, any trend data on hypospadias should be viewed with considerable caution.

A descriptive epidemiological study, which utilized data from various malformation surveillance systems (Hungary, Sweden, Denmark, Italy, Spain, South America, and Mexico), demonstrated an increased prevalence of hypospadias (Källén et al., 1986). There was considerable variation in the incidence reported for the different systems, with South America and Mexico reporting the lowest rates and Hungary and Sweden the highest for the years 1980–1981. Misdiagnosis was evaluated in this study and found to

Table 5.5 - Examples of Endocrine Effects in Experimental Male Animals Exposed to Endocrine Disruptors during the Prenatal/Perinatal Period*

Chemical	Mode of Action	Species and Exposure Period	Doses and Route	Effects**	Reference
E_2	ER agonist	Rat One generation	0.05–50 ppm in diet (0.003–4.12 mg/kg/d)	At 10 and 50 ppm: reduced weight testes and epi didymides; atrophy testes and epididymides; seminiferous tubule degeneration; reduced sperm numbers, motility; no effect on Sertoli cell number	Biegel et al.,1998; Cook et al., 1998
E_2	ER agonist	Mouse GD 13–19	25–300 µg/mouse subcutaneously	At 25 and 100 µg: increased prostate weight	Welshons et al., 1999
17α-Ethinyl estradiol	ER agonist	Mouse GD 0–17	0.002–200 µg/kg/d orally	At 0.02–2 µg/kg/d: increased prostate weight	Thayer et al., 2001
DES	ER agonist	Mouse GD 11–17	0.02–200 µg/kg/d orally	At 0.02–2 µg/kg/d: increased prostate weight	Vom Saal et al., 1997
Bisphenol A	Weak ER agonist	Mouse GD 11–17	2, 20 µg/kg/d orally	At 2 and 20 µg/kg/d: increased prostate weight	Nagel et al., 1997
Nonylphenol	Weak ER agonist	Rat PND 1–18	0.08–8 mg/kg/d intraperitoneally	At 0.8 mg/kg/d: reduced testis, epididymis, seminal vesicle and prostate weight At 8 mg/kg/d: Reduced anogenital distance, cryptorchidism, poor differentiation of seminiferous tubules, reduced in sperm count, motility and fertility	Lee, 1998; Lee et al., 1999
Methoxychlor	Metabolite is ER, antagonist AR agonist	Mouse GD 11–17	20, 2,000 µg/kg/d orally	At 20 and 2,000: µg/kg/d increased prostate weight	Welshons et al., 1999
Methoxychlor	Metabolite is ER agonist, AR antagonist	Rat One generation	25–200 mg/kg/d	At 25 mg/kg/d: reduced growth At 50 mg/kg/d: reduced caudal sperm count At 100 mg/kg/d: delayed puberty	Gray et al., 1989
Methoxychlor	Metabolite is ER agonist, AR antagonist	Rat GD 14– PND 21 or 42	5–150 mg/kg/d by gavage	At 50 mg/kg/d: reduced growth, delayed puberty, reduced testis ,epididymis, seminal vesicle and prostate weight At 150 mg/kg/d: reduced caudal sperm count, reduced sperm motility	Chapin et al., 1997a
DDT	ER agonist	Mouse GD 11–17	18, 180 µg/kg/d	At 18 and 180 µg/kg/d: small testes, altered aggressive behavior, altered territorial behavior	Vom Saal et al., 1995; Palanza et al., 1999
p,p'-DDE	Weak AR antagonist	Rat GD 14–18	10 or 100 mg/kg/d by gavage	At 100 mg/kg/d: reduced anogenital distance, retained nipples, hypospadias, reduced prostate, glans penis and cauda epididymis wt, prostatic atrophy and prostatitis	Kelce et al., 1995; You et al., 1995; Gray et al.,1999b
Vinclozolin	Metabolites AR antagonists	Rat GD 14– PND 3	3–200 mg/kg/d by gavage	At 3 mg/kg/d: reduced anogenital distance, retained nipples At 50 mg/kg/d: cleft phallus and hypospadias, suprainguinal testes, vaginal pouches, epididymal granulomas, reduced sperm count and fertility At 100 mg/kg/d: small to absent sex accessory glands	Gray et al., 1994, 1999a
Procymidone	AR antagonist	Rat GD 14–PND 3	25–200 mg/kg/d by gavage	At 25 mg/kg/d: reduced anogenital distance At 50 mg/kg/d: retained nipples, hypospadias, vaginal pouch, reduced prostate and seminal vesicle wt, prostatic atrophy and prostatitis	Ostby et al., 1999; Gray et al., 1999b
Linuron	AR antagonist	Rat GD 12–21 GD 14–18	By gavage: 12.5–50 mg/kg/d or 100 mg/kg/d	At 12.5 mg/kg/d: retained nipples, hypoplastic testes At 100 mg/kg/d: reduced anogenital distance, retained nipples, hypospadias, reduced testis, prostate, glans penis, cauda epididymis and epididymides weight, testicular and epididymal atrophy	Gray et al., 1999b; Lambright et al., 2000; McIntyre et al., 2000a, 2000b
Linuron	AR antagonist	Rat One generation	10–40 mg/kg/d by gavage	At 40 mg/kg/d: delayed puberty	Gray et al., 1999b
Dibutyl phthalate	Reduced T in fetal testis	Rat Continuous breeding	0.1–1% in the diet (50–800 mg/kg/d)	At 1% in diet (800 mg/kg/d): Testicular degeneration, epididymis absent/underdeveloped, reduced number of spermatids, reduced mating, fertility	Wine et al., 1997
Dibutyl phthalate	Reduced T in fetal testis	Rat GD 11–21	0.5–2% in the diet (330–660 mg/kg/d)	At 1% in diet (555 mg/kg/d): reduced anogenital distance, undescended testes	Ema et al., 1998
Dibutyl phthalate	Reduced T in fetal testis	Rat GD 3–PND 21 or GD 12–21	100–750 mg/kg/d by gavage	At 250 mg/kg/d: reduced anogenital distance, retained nipples, hypospadias, delayed puberty, epididymis absent/underdeveloped, atrophied seminiferous tubules, reduced spermatogenesis At 500 mg/kg/d: reduced testis weight, absent prostate and seminal vesicles	Mylchreest et al., 1998, 1999; Gray et al., 1999b; Mylchreest et al., 2000
Diethylhexyl phthalate	Reduced T in fetal testis	Rat GD 14–PND 3	750 mg/kg/d by gavage	Reduced anogenital distance, retained nipples, hypospadias, vaginal pouch, reduced testis, prostate, glans penis, cauda epididymis and epididymides weight, testicular and epididymal atrophy	Gray et al., 1999b; Parks et al., 2000
2,3,7,8-TCDD	AhR agonist	Rat GD 15	0.05–1 µg/kg	At 0.05 µg/kg: reduced sperm count At 0.2 µg/kg: delayed puberty At 1 µg/kg: reduced anogenital distance	Gray et al., 1997a, 1997b

*For some of the low-dose effects cited above, other (uncited) authors have been unable to replicate the effects. **Doses in this column indicate lowest dose at which effect seen. Reduced T, reduced synthesis of testosterone.

occur in varying degrees in the different regions. Correction for underascertainment resulted in comparable incidence rates for Denmark and Sweden, two countries with similar social, economic, and reproductive practices as well as pollution levels. If persistent environmental chemicals were contributing to the prevalence of hypospadias, then one would predict that because body burdens increase with age but breast-feeding significantly reduces the maternal body burdens: 1) the incidence of hypospadias should be greater in first-born males, 2) the prevalence will decrease with parity, 3) maternal age at first pregnancy should be associated with an increased prevalence, and 4) zygosity should not contribute meaningfully to the prevalence. In the study of Källén et al. (1986), there was an increased prevalence of hypospadias in first-born males, the prevalence decreased with parity, and maternal age was associated with an increased prevalence. Moreover, although more common in monozygous twins compared to dizygous, the difference was not significant. Two birth defects surveillance systems in the USA also indicated that the prevalence of hypospadias at birth has increased between the 1970s and 1990s (Paulozzi et al., 1997).

However, in England and Wales, the incidence of hypospadias appears to be decreasing following a steady increase between 1965 and 1983 (MRC, 1995). Studies in Finland also have not shown any increase in the incidence of hypospadias. An analysis of the national hospital discharge registry of 1,543 boys born between 1970 and 1986 and surgically treated for hypospadias before 9 years of age from a total of 549,176 male births showed that the prevalence of hypospadias was constant over this time period (Aho et al., 2000). Another study of hypospadias diagnosed at birth in Turku, Finland (Virtanen et al., 2001), found no increase in the rate (0.3%) in a cohort of 5,798 boys born between 1997 and 1999, compared with the rate found by Aho et al. (2000) or compared with the nationwide birth rate of hypospadias during 1993 to 1998. Aho et al. (2000) also discuss the possibility of apparent regional/temporal differences in hypospadias being due to completeness of recording methods.

Analysis of the temporal trends in the prevalence of cryptorchidism also indicates an increase over time. The prevalence of cryptorchidism was investigated by cohort analysis of patient discharge records for the years 1962–1981 for England and Wales (Chilvers et al., 1984) and demonstrated an increase in the prevalence of undescended testis from 1.4% for the 1952 birth cohort to 2.9% for the 1977 birth cohort. This study relied on the rate of orchiopexies carried out each year, and thus it is possible that the entire increase in the described prevalence could be accounted for by a change in the criteria used to select patients for surgery.

In a prospective study (Ansell et al., 1992), 7,441 boys from Oxford were examined for cryptorchidism at birth and then again at 3 months of age during 1984–1988. The cryptorchidism rate at birth was found to have increased by 35.1%, and at 3 months of age by 92.7%, compared with the rates reported for the mid-1950s in 3,612 male infants in London by Scorer (1964). Although direct comparison between these two studies is hampered by different inclusion criteria, because Scorer (1964) included stillbirths and low-birth-weight babies, both of which would increase the incidence relative to the later study, it would seem that the prevalence of cryptorchidism has increased in Great Britain.

A more exhaustive analysis of the trends of both hypospadias and cryptorchidism has recently been reported (Paulozzi, 1999). The birth prevalence rates for hypospadias and cryptorchidism were collected through the International Clearing House for Birth Defects Monitoring System. The rates were systematically and continuously collected in 29 registries from 21 countries recording a total of 4 million births per year. A wide intercountry variation in rates of hypospadias and cryptorchidism around the world was found. A factor of 3 or more could be observed between the highest rates (in USA and Israel for hypospadias, USA and Canada for cryptorchidism) and the lowest rates (Finland, Japan, China, and South America for hypospadias; South America for cryptorchidism). However, differences in methodologies and other factors make comparisons difficult. Although the temporal evolution within various registries suggests an increase in hypospadias rates during the 1970s and 1980s in the USA, Scandinavia, and Japan, no change was observed in Canada. No clear significant increase in cryptorchidism prevalence was observed. For both pathologies a tendency toward a decline of rates was found after 1985.

5.1.6.4.3 Influence of environmental chemicals. In animal experiments, cryptorchidism has been induced with gestational exposure to suspected estrogenic and antiandrogenic chemicals, such as mono-*n*-butyl phthalate in rats (Imajima et al., 1997) and flutamide in pigs (McMahon et al., 1995). Midgestational exposure to TCDD has produced cryptorchidism, reduced germ cell numbers, and epididymal abnormalities in pigs, accompanied by reduced ERα mRNA expression in the gubernaculum and epididymis and increased ERα protein levels in the testis (Barthold et al., 1999). Other experimental examples are presented in Table 5.5 and in Chapter 3 (section 3.12). These all suggest that chemicals with estrogenic or antiandrogenic activity can induce cryptorchidism and hypospadias.

A number of epidemiological studies have suggested that exposure to pesticides may be linked to male reproductive tract abnormalities. An increased rate of orchiopexy has been reported in areas with extensive use of pesticides in intensive farming in Granada, Spain (Garcia-Rodríguez et al., 1996). An increased risk of cryptorchidism and possibly hypospadias has also been reported in Norwegian boys born on farms where pesticides were being used (Kristensen et al., 1997). Increases in urogenital defects have also been noted in children born to those occupationally exposed to pesticides in Colombia (Restrepo et al., 1990) and in Minnesota, USA (Garry et al., 1996). Møller and Weidner et al. (1998) analyzed data from all live male infants discharged from Danish hospitals with a diagnosis of cryptorchidism or hypospadias between 1983 and 1992 and found a significantly increased risk of cryptorchidism but not hypospadias in sons of women working in gardening (OR, 1.7; 95% CI, 1.1–2.4). However, the association accounted for a very small proportion of the total number of cryptorchidism cases (0.3%). No increased risk was found in sons of men working in gardening or farming.

The possible role of the maternal diet in hypospadias has recently been reported (North and Golding, 2000). In this longitudinal population-based study there were 7,928 boys, from which there were 51 hypospadias cases identified by maternal reporting using the records for referral and/or surgery, birth notifications, records of examination by neonatal pediatricians, and post mortem reports. Mothers who were vegetarian in pregnancy had an increased risk of giving birth to a boy with hypospadias compared with omnivores who did not supplement their diet with iron (unadjusted OR, 4.99; 95% CI, 2.10–11.88). Omnivores who supplemented their diet with iron in the first half of pregnancy also had a raised risk (unadjusted OR, 2.07; 95% CI, 1.00–4.32). However, the increased risk was obviated when adjusted ORs were

calculated. Women who reported having influenza during the first trimester also had an increased risk of giving birth to a boy with hypospadias (unadjusted OR, 3.39; 95% CI, 1.50–6.78). It was suggested that vegetarians would have a greater exposure to phytoestrogens than omnivores and this might explain the raised risk in that group. In addition to weaknesses surrounding identification of hypospadias cases, no data are available concerning the amount of phytoestrogens that were consumed by the women or to which the developing fetus was exposed. Other potential sources of exposure were not evaluated in this study.

5.1.6.4.4 Endocrine influences. The development of the male reproductive tract is under sex hormone control (section 5.1.8). Hypospadias and cryptorchidism could therefore be considered as likely markers of endocrine disturbance. Animal studies have demonstrated that exposure to estrogens during development can result in cryptorchidism and hypospadias (Grocock et al., 1988; Vorherr et al., 1979). In humans, the induction of reproductive tract abnormalities (epididymal cysts, cryptorchidism, and other genital abnormalities) in sons of DES-exposed mothers have been well documented (Henderson et al., 1976; Wilcox et al., 1995), but it should be noted that prenatal DES exposure was not associated with hypospadias. However, a meta-analysis of 14 well-selected human studies on the influence of exogenous hormones (oral contraceptives, hormone pregnancy tests, progestagens used in threatened abortion or because of previous miscarriages) has not produced any convincing evidence of an effect of prenatal exposure (Raman-Wilms et al., 1995).

Mutations in the gene coding for the AR are also unlikely candidates as causative factors to explain most cases of either cryptorchidism or hypospadias for the majority of cases, due to the rarity of their occurrence in affected children (Bentvelsen et al., 1995; Sutherland et al., 1996). Lower circulating levels of testosterone have however been demonstrated during gestation weeks 6–14 in boys with cryptorchidism (Garcia-Rodriguez et al., 1996). It is considered plausible that environmental contaminants that increase testosterone turnover, or decrease androgen synthesis, or antagonize androgen action at the receptor level could contribute to an increased prevalence of cryptorchidism.

5.1.6.5 The prostate.

5.1.6.5.1 Effects of developmental estrogenic exposure. The rat prostate undergoes branching morphogenesis after birth (Hayashi et al., 1991) and can be imprinted by endogenous and exogenous hormones during that period (Rajfer and Coffey, 1979). Rajfer and Coffey found that a brief neonatal estrogen exposure permanently imprints prostatic development and is associated with an increased incidence of hyperplasia, dysplasia, and adenocarcinoma with aging. The critical period for persistent effects of estrogen on the human prostate appears to be up to as late as PND 5 and begins before birth (Higgins et al., 1981). Further investigations by Bellido and coworkers (1985) found that males that had been injected with 500 µg of E_2 benzoate on PND 1 exhibited ventral prostate and testicular atrophy, decreased testosterone levels, and a transient increase in prolactin plasma levels on PNDs 15 and 22. Reduced androgen sensitivity and AR levels in adulthood in the ventral and dorsal lobes were also observed following treatment with 25 µg E_2 on PNDs 1, 3, and 5 (Prins, 1987). Therefore, estrogen can block differentiation pathways during development, and this is mediated in part by a block in AR expression in the epithelial and smooth muscle cells.

5.1.6.5.2 Effects of prolactin and E_2 on adult lateral prostate growth. It is well known that estrogen administration in an adult male rat can result in both the regression and growth of the prostate. However, the difference in effect is related to the age and hormonal status of the animal, in addition to the dose of estrogen administered. Estrogen exposure of an intact male results in atrophy of the prostate, which is an indirect effect caused by the suppression of anterior pituitary gonadotropin secretion and subsequent reduction or complete suppression of testosterone secretion (Price and Williams-Ashman, 1961; Mawhinney and Newbauer, 1979). In contrast, estrogen exposure to castrated rats results in an increase in growth, with stromal hypertrophy (Thompson et al., 1979) or epithelial hyperplasia (Salander and Tisell, 1976).

Following E_2 administration in the adult rat, increases in weight, citric acid concentration (Grayhach and Lebowitz, 1967; Walvoord et al., 1976), zinc accumulation (Gunn and Gould, 1956), and uptake of androgen (Tveter and Aakvaag, 1969) were observed only in the lateral lobe. Because prolactin can produce the same effects on the lateral prostate as estrogens in rats (Grayhach and Lebowitz, 1967; Moger and Geschwind, 1972; Negro-Vilar et al., 1977; Holland and Lee, 1980), the effects of estrogen on the stimulation of prostatic growth were believed to be due to increases in the production of pituitary prolactin, because estrogen causes a marked release of prolactin from the pituitary gland (Meites et al., 1972).

Prolactin has been repeatedly shown to stimulate prostatic growth (Grayhach and Lebowitz, 1967; Thomas and Manandhar, 1975; Negro-Villar et al., 1977; Holland and Lee, 1980; Prins and Lee, 1983) and to delay prostatic regression (Kolbusz and Grayhack, 1982). A direct prolactin stimulation of the lateral prostate has been demonstrated using pituitary grafts in castrated males to increase prolactin secretion (Prins, 1987; Schact et al., 1992). However, in these studies the ventral and dorsal lobes were unaffected. This effect was also seen in an *in vitro* study, in which prolactin increased DNA synthesis but only in the lateral prostate (Nevalainen et al., 1991).

The effect of prolactin on the growth of the lateral prostate and the relationship of this effect with androgens was then investigated. Prins (1987) found that prolactin increased endogenous DHT binding to the AR and induced an increase in nuclear uptake of ^{3}H-DHT. It was proposed that there was an increased quantity of androgen-binding sites when serum prolactin is elevated. Prins (1987) also found that the hormonal regulation of ARs differs in the lateral lobe as compared with the ventral and dorsal lobes. The lateral lobe has an androgen-independent component of AR protein expression whereas that of the ventral and dorsal lobes is androgen dependent. Adult exposure to estrogens down-regulates AR expression in the ventral prostate but has no effect on those receptors in the other lobes. Prolactin up-regulates ARs in the lateral prostate specifically and is associated with enhanced growth and secretory activity within that lobe.

5.1.6.5.3 Prostatitis. Prostatitis without a known bacterial origin (nonbacterial prostatitis) is fairly common and poorly defined in humans and is particularly troubling in young and middle-aged men because it can affect fertility (Meares, 1998). A prostatic inflammatory infiltrate is also found in many cases of benign prostatic hyperplasia and prostate cancer (McClinton et al., 1990). Histologically, some strains of aged rats develop a spontaneous prostatitis in their lateral prostates, with an accumulation of neutrophils in the lumina and mononuclear cells in the stroma (Aumuller et al., 1987). In 1988, Robinette showed that E_2 treatment could induce an inflammatory response in the lateral prostate after a 2-week E_2 exposure in the 1-week castrate adult male rat. The existence of hormone-induced inflammation on a long-term basis was associated with subsequent thickening of the

fibromuscular stroma that resembled changes seen in the lateral prostate of the aging rats mentioned above. The presence of this inflammation was correlated with an increase in serum prolactin (Tangbanluekal and Robinette, 1993). The administration of bromocriptine to these E_2-treated males suppressed serum prolactin levels and the inflammatory response seen in the lateral prostates, indicating that prolactin mediated the response (Tangbanluekal and Robinette, 1993). To further characterize this effect, the investigators also found a dose–response relationship between the administration of exogenous prolactin and the severity of the inflammation that was induced.

Estrogen administration to rats postnatally has also been shown to result in ventral prostate inflammation in adulthood (Rajfer and Coffey, 1978; Prins, 1997; Naslund et al., 1988). Other histologic changes that have been observed in adult prostate following neonatal estrogenization include hypotrophic and disorganized epithelium and an increase in stromal elements (Rajfer and Coffey, 1978; Prins, 1997).

Recently, it has been shown that environmental exposures to certain pesticides and environmental compounds during the perinatal, lactational, or early pubertal periods can lead to lateral prostate inflammation or prostatitis in the adult male rat (Stoker et al., 1999a, 1999b, 1999c). These exposures appear to be increasing the secretion of prolactin prior to puberty, which in turn seems to have a relationship with the level of prostatitis observed in the adult male rat.

5.1.6.5.4 Prostate weight and low-dose issues. *In utero* exposures to environmental compounds that have been shown to possess estrogenic activity, such as DES, can induce persistent structural and functional alterations in the developing reproductive tract of male mice (Santti et al., 1998). Recently, there has been much debate over exposure to low doses versus higher toxicological doses of environmental estrogens during the latter half of gestation. Lower doses of bisphenol A and DES *in utero* have been found to increase prostate weight in adulthood, whereas higher doses of the same compounds result in the opposite effect, a decreased prostate weight (Gupta, 2000; vom Saal et al., 1995, 1997; Nagel et al., 1997) (Table 5.5). However, when attempts were made to repeat these studies, none of the adverse effects of low doses of bisphenol A and DES were observed (Cagen et al., 1999; Ashby et al., 1999).

5.1.6.6 Conclusions on male reproductive tract abnormalities. The data on temporal trends in the incidence of hypospadias and cryptorchidism should be interpreted with considerable caution, given the lack of longitudinal studies and the consequent difficulties in comparing data from separate studies in which definition, ascertainment, and registration of these defects may have differed substantially. Data on trends in pathology and possible endocrine-mediated disturbances on the prostate in men, other than cancer, are lacking, although there is now considerable experimental evidence that hormones and other endocrine-active chemicals can affect prostate development.

Studies on environmental chemicals have focused mainly on pesticides. However, most of the human studies have included only small numbers of cases occupationally exposed to pesticides and lacking good exposure data. In some studies, the increase in urogenital defects was part of a general increase in birth defects amongst offspring of pesticide-exposed parents. No conclusions can be drawn on whether there is a causal association with pesticide exposure or whether there is endocrine involvement. However, animal data on several EDCs and on sex hormones and human and animal data on DES clearly demonstrate that hormonal mechanisms can be involved in the etiology of male reproductive tract abnormalities, and the impact of environmental chemicals certainly requires further study.

5.1.7 Endometriosis

5.1.7.1 Pathobiology and the role of estrogen. Endometriosis is an estrogen-dependent disease characterized by the presence of endometrial glands and stroma outside the uterine cavity. It is a common gynecologic disorder as well as a major cause of infertility (Chedid et al., 1995) affecting approximately 14% of women of all reproductive ages (Vercillini et al., 1995). Proliferation of a progenitor stem cell (Meyer, 1897, 1925), or retrograde menstruation of endometrial cells (Sampson, 1927, 1940), or substances shed from the uterine cavity inducing the undifferentiated cells of the peritoneum to undergo endometrial differentiation are thought to lead to the implantation and proliferation of ectopic endometrial cells. At the present time, it is generally appreciated that there is no one single theory that identifies and can explain all aspects of the clinical syndrome. Although retrograde menstruation and bleeding into the peritoneal cavity during menstruation are widely accepted as major contributing factors in the pathogenesis of this disease, it is a common phenomenon even in women without endometriosis (Halme et al., 1984). Hence, factors other than retrograde menstruation are thought to contribute to the development and progression of endometriosis.

There is a clear clinical relationship of endometriosis to endogenous and exogenously administered estrogen and progesterone exhibiting a dose–response effect. The exogenous administration of estrogen has been shown to aggravate the disease. It can be speculated that imbalance of estrogen and progesterone may be involved in the pathogenesis and pathophysiology of this disease. There is evidence that the incidence is reduced through use of oral contraceptives, apparently associated with the inclusion of progesterone and the reduced amount of uterine bleeding. It has been hypothesized that the steady reduction in the estrogen content of the oral contraceptive has resulted in a greater progestin effect that results in a reduced incidence of endometriosis. There is an association of the onset of the disease and progression of the disease with continued menstrual function. The disease can undergo a reduction in symptoms in association with pregnancy but often recurs with the re-establishment of menstrual function. The cessation of menstrual function at menopause is associated with virtual elimination of the disease. Thus, although there is a clear association of severity with estrogen, and it is clear that endometriosis never occurs in its absence, it is generally accepted that the disease is not caused specifically by estrogen but is stimulated by its presence (Guarnaccia and Olive, 1998).

5.1.7.2 Evidence from chemical exposures in humans. Chemical contaminants have been implicated in the pathobiology of endometriosis as a result of a series of clinical-observational and animal studies. In addition, AhR expression has been demonstrated in human endometrium throughout the menstrual cycle (Igarashi et al., 1999). In humans, an association between endometriosis and exposure to PCBs (Gerhard and Runnebaum, 1992) and dioxins (Koninckx, 1999) has been made. A positive association between endometriosis and dioxin exposure was also reported in a single case–control study in which 44 women with endometriosis were compared with 35 age-matched controls with tubal infertility (Mayani et al., 1997). Although significantly more women with endometriosis [8 (18%), vs. 1 woman (3%) of the control group]

tested positive for dioxin in their serum (p = 0.04), there was no relationship between severity of endometriosis and concentration of dioxin. Moreover, although an OR of 7.6 was obtained, the 95% CI included unity (0.87–169.7). In another case–control study, no association between plasma organochlorine concentrations and endometriosis could be found in 86 women with endometriosis compared with 70 controls, matched for the indication of laparoscopy (Lebel et al., 1998). A study of patients presenting for pelvic pain, infertility, and requests for sterilizing tubal fulguration was done to compare the plasma concentrations of organochlorines among those with and without endometriosis. There were no differences found between those with the disease and those without, although there were no power considerations to determine the strength of this negative study (Lebel et al., 1998). The most significantly exposed women in Seveso also underwent an evaluation for endometriosis in a case–control study, which showed there was no association between dioxin levels and the presence or amount of endometriosis in women (Mocarelli et al., 1999). Similarly, a preliminary study of 15 women with endometriosis and 15 controls did not find a statistically significant association with serum levels of PCBs or dioxin (Boyd et al., 1995). These studies are all relatively small, and thus may not have the statistical power to detect differences if they were indeed present. It has been determined that a sample size of 286 subjects with endometriosis and 286 control subjects would be required to detect a twofold increase in the incidence of endometriosis, assuming a 10% prevalence rate, significance level of 0.05 and power level of 90% (Mayani et al., 1997). There are no direct data regarding the impact of phytoestrogens, such as isoflavones or flavanones, on ectopic human endometrium, although it could be anticipated that they might have an effect, given that soy isoflavones inhibit E_2-mediated endometrial proliferation in macaque monkeys. Hence, the human data at present neither confirm nor refute the hypothesis that environmental contaminants play a role in the pathobiology of endometriosis.

5.1.7.3 Evidence from animal studies. The suggested role of dioxins in the pathobiology of endometriosis derives also from a variety of animal experiments. Most notable of the animal studies is a reproduction study in which rhesus monkeys were dosed with TCDD in the diet for 4 years (Rier et al., 2001). Severe endometriosis was discovered in a couple of animals, necessitating necropsy and leading the study team to examine the remaining animals by laparoscopy for the presence of this disease. When the study code was revealed, the prevalence of endometriosis was found to be 33%, 43%, and 71% respectively for monkeys that received 0, 5, and 25 ppt TCDD. These data therefore suggest that TCDD may play a role in the development of endometriosis, although the mechanism was not explored in this study. However, this study has been severely criticized by Golden et al. (1998), and the complexities associated with the use of rhesus monkeys to study this disease have been discussed by McCann and Myers (1970). For example, there is a higher prevalence of endometriosis in rhesus monkeys than in women. In this study, the background rate of endometriosis was identified by historical autopsy records, whereas the incidence in treated animals was determined by laparoscopy. Other factors may also have confounded the results, such as the relative rates of cesarean section, a procedure that increases the rates of endometriosis in monkeys. Although an immune mechanism has been proposed (section 5.4), no data were presented to indicate that these animals could be characterized as having immune suppression. Despite the limitations detailed above, the potential contribution of TCDD to the pathobiology of endometriosis cannot be discounted. Rier et al. (2001) have published further information on the monkeys in their original study, showing that serum levels of TCDD and specific dioxinlike PHAH congeners were increased in TCDD-treated animals with endometriosis 13 years after the TCDD exposure. The animals with high serum levels of the congeners 3,3′,4,4′-tetrachlorbiphenyl and 3,3′,4,4′,5-pentachlorbiphenyl and increased total serum TCDD equivalents (TEQ) had a high prevalence of endometriosis and the severity of the disease correlated with the serum concentration of 3,3′,4,4′-tetrachlorbiphenyl. Other studies have also followed the original study of Rier et al. (2001) in an effort to clarify the role of various chemical contaminants in the etiology of endometriosis.

In a reproductive/developmental toxicology study, no association could be demonstrated between the incidence of endometriosis in rhesus monkeys and treatment with Aroclor 1254 (Arnold et al., 1996). In another study on cynomolgous monkeys (Yang et al., 2000), TCDD treatment (0, 1, 5, and 25 ng TCDD/kg body weight/day) induced a bimodal effect on endometrial implant survival and size. Circulating gonadal steroid levels and menstrual cycle characteristics were unchanged by treatments in this study, and no data were provided on the direct effects of TCDD on the endometrium or immune function.

Although rodents do not spontaneously develop endometriosis, surgical induction of endometriosis in rodents was enhanced by exposure to TCDD (Johnson et al., 1997). The significance of these studies for humans is uncertain. In particular, it should be noted that the effects occurred with relatively high doses of TCDD. Prenatal treatment on GD 8 (3 or 10 µg/kg by gavage), followed by further TCDD treatment (3 or 10 µg/kg by gavage) as adults prior to surgical induction of endometriosis also increased the size of endometriotic lesions in mice but not in rats (Cummings et al., 1999).

In a similar murine model, ovariectomized and controlled for estrogen replacement, administration of 4-chlorodiphenyl, an estrogenic compound, was associated with increased endometriotic cyst growth (Yang et al., 1997). In contrast, Yang and Foster (1997) have shown that the administration of TCDD inhibited the growth of endometriotic cysts in the presence of estrogen and indicated that the experimental model did, however, implicate TCDD as an agent that could influence tissues that are responsive to estrogen. TCDD is a potent AhR ligand. AhR ligand binding affects a number of genes that are potentially important in the pathobiology of endometriosis. In particular, AhR ligand binding regulates EGF, interleukin-1β, TGF-α and TGF-β (Madhukar et al., 1984; Sutter et al., 1991; Gaido et al., 1992). TCDD additionally inhibited the E_2-induced increase in uterine wet weight in the rat (Gallo et al., 1986) and in mice the E_2-induced increase in uterine EGF mRNA levels. Contradictory reports on the effect of TCDD on uterine ER levels; TCDD-induced suppression in rats have been reported (Romkes et al., 1987; Astroff et al., 1990), whereas others found no effect in mice (De Vito et al., 1992). However, decreased expression of ER mRNA in the ovaries and uteri of TCDD-treated mice has recently been demonstrated (Tian et al., 1998).

Molecular mechanisms of TCDD action have been investigated in a nude mouse model bearing implants of human endometrial tissue (Bruner et al., 1997). Human endometrial explants cultured with E_2 were found to secrete stromal- and epithelial-specific matrix metalloproteinases and induced ectopic endometrial lesions when injected into nude mice. However, inclusion of progesterone with E_2 suppressed matrix metalloproteinase secretion *in vitro* and lesion formation *in vivo* (Bruner et al., 1997; Bruner-Tran et al., 1999).

The combination of E_2 and TCDD increased the number and size of endometriotic lesions compared to E_2 alone, whereas TCDD blocked the ability of progesterone to suppress the matrix metalloproteinase secretion and lesion formation *in vivo* (Bruner et al., 1997; Bruner-Tran et al., 1999).

5.1.7.4 Conclusions on endometriosis. In summary, the evidence shows that endogenous and pharmacological doses of estrogens are potential modifiers of endometriosis. However, there are severe constraints on the biological plausibility that TCDD, the main environmental chemical investigated, enhances the disease because it has antiestrogenic effects and exposures would be exceedingly small compared with the high doses used in rodent experiments. One controversial primate study implicating lower levels of TCDD in enhancement of the disease needs further corroboration. Although the study of the survivors of Seveso exposed to high levels of TCDD was negative, this and other human studies were all small, indicating that more powerful analyses are required. Possible mechanisms linking the endocrine and immune systems in this disease also require further study.

5.1.8 Other Adverse Reproductive Outcomes Potentially Linked to EDCs

Environmental chemicals have also been suggested as potential causative factors in the temporal decline in the age of onset of puberty, in polycystic ovarian syndrome, and in shortened lactation.

5.1.8.1 Precocious puberty. The development of secondary sexual characteristics in girls before 8 years of age or in boys before 9 years of age is considered to meet the criteria for a diagnosis of precocious puberty. The current classification of the disorder recognizes 1) central precocious puberty, 2) peripheral precocious puberty, and 3) contrasexual precocious puberty (Bates, 1998). Thus, the hormonal mechanisms are better understood because the abnormal condition is similar to normal physiological developments. Population-based studies showing a reduction in the median age of puberty demonstrate the need for investigations of the possible causes of this trend.

A hypothesized external agent that would induce central precocious puberty would act through the early initiation of the pulsatility of gonadotropin-releasing hormone from the hypothalamus, thereby inducing the cascade of hormonal events that result in pubertal development. However, review of the literature has not established a relationship between central precocious puberty and environmental agents.

In the case of peripheral precocious puberty, the mechanism in girls is through hormonal receptors in peripheral tissues that are responsive to estrogen or estrogenlike compounds. Several reports have indicated there is an association between premature breast development and exposure to DES (Hertz 1979), ethinyl estradiol, and mestranol. This finding has been reported in association with exposure to contaminated meat (Fara et al., 1979; Kimball et al., 1981). DES has been found as a contaminant of baby food containing veal and poultry (Hoffmann, 1982). In Puerto Rico, reports of precocious puberty that have heightened concern that there may be an environmental cause (Mills et al., 1981; Nizzoli et al., 1986; Freni-Titulaer et al., 1986; Hannon et al., 1987; Van Winter et al., 1990). For example, a temporal trend toward premature breast development (premature thelarche) in girls and gynecomastia in boys has been noted in Puerto Rico during the early 1980s. Concerns were expressed that this was due to increased estrogenic compounds from the environment (Mills et al., 1981; Bongiovanni, 1983; Saenz de Rodriguez et al., 1985; Freni-Titulaer et al., 1986). The possible sources considered were foodstuffs and also the presence of waste products from pharmaceuticals because of the high levels of local industrial pharmaceutical production. In a recent report (Colon et al., 2000), serum samples from 41 girls with premature breast development and 35 controls were analyzed for pesticides and phthalate esters. No pesticides or their metabolites could be found in any of the serum samples; however, 28 (68%) of the girls with premature breast development had measurable levels of phthalates [dimethyl, diethly, dibutyl and di-(2-ethylhexyl)], compared with 6 of the 35 (17%) control samples. Although these data suggest that phthalate plasticizers may be associated with precocious breast development in this population, detection of diesters of phthalates in serum would not be expected because they are normaly rapidly metabolized to their respective monoesters before absorption. This and the small sample size suggest that these results should be interpreted with caution. Another study, in which plasma of girls with precocious puberty was screened for pesticides, showed an increase in *p,p′*-DDE in foreign children with precocious puberty immigrating from developing coutries to Belgium, compared with nondetectable levels in Belgian-born girls with idiopathic or organic precocious puberty, suggesting a possible relationship to early exposure to *p,p′*-DDE (Krstevska-Konstantinova et al., 2001).

The effect of *in utero* exposure to PBBs on sexual maturation was evaluated in Michigan girls whose mothers were accidentally exposed through the diet (Blanck et al., 2000). In 1973, the flame retardant FireMaster was inadvertently added to livestock feed instead of the nutritional supplement NutriMaster. Michigan residents subsequently consumed animals and dairy products contaminated with PBBs. Exposure was estimated on the basis of maternal blood samples collected years after the initial exposure. Effects on pubertal end points were assessed by questionnaires sent to mothers of daughters less than 18 years of age and to the daughters themselves. The data reveal that menarche and pubertal hair growth were significantly advanced in girls with high perinatal exposure [unadjusted OR and CI, 0.9, 0.4–1.8 (not breast fed) and 2.1, 0.9–5.3 (breast fed), 0.6, 0.1–2.7 (not breast fed) and 8.4 1.4–50.5 (breast fed), respectively; adjusted OR and CI, 0.8, 0.3–1.9 (not breast fed) and 3.4, 1.2–9.0 (breast fed), and 0.9, 0.2–4.3 (not breast fed) and 19.5, 2.8–138.2 (breast fed), respectively]. There was no association with Tanner stage of breast development. These findings are both interesting and perplexing because menarche and breast development are estrogen dependent whereas pubertal hair growth is dependent. This study suffers from a number of limitations, most notably, the weak exposure assessment that is prone to missclassification error. No firm conclusions can be drawn.

Precocious sexual maturation in rodents has been studied by examining experimental animals for preputial separation in males or premature vaginal opening in females. For example, the age of vaginal opening in Long-Evans rats was advanced by oral exposure to estrogenic chemicals (Laws et al., 2000b). Specifically, advanced age at vaginal opening was found in rats treated orally on PNDs 21–35 with ethynyl estradiol (0.01 mg/kg), methoxychlor (50 mg/kg), 4-*tert*-octyphenol (200 mg/kg), or 4-nonylphenol (50 mg/kg). It does not necessarily follow that exposure to estrogenic compounds will induce precocious puberty as was shown by treatment with bisphenol A, which induces estrogenic responses in the uterotropic assay but at doses up to 400 mg/kg failed to advance vaginal opening (Laws et al., 2000a).

5.1.8.2 PCOS. PCOS, a poorly understood condition, has been described as a self-perpetuating state of chronic anovulation. There is a broad variation of clinical findings ranging from the

classical Stein-Leventhal syndrome to much milder forms of anovulation (Stein and Leventhal, 1935; Ben Shlomo et al., 1995). The syndrome is usually associated with the onset of puberty. The menses may at first be ovulatory, but shortly thereafter oligomenorrhea or amenorrhea ensues. Mild to moderate hirsutism may develop, and obesity is common. The concomitant use of oral contraceptives may delay or reduce the appearance of symptoms. The actual incidence is not established because of the great variation in presentation and the very real possibility of underdiagnosis. However, Polson et al. (1988) have indicated through ultrasound evaluation that polycystic ovaries may be a feature in 22% of young women. The interest in this syndrome in relation to environmental chemicals stems from the animal literature. There is evidence that testosterone exposure has significant effects on the female developing brain, including induction of acyclicity and anovulation (Chapter 3, section 3.3.5). Polycystic ovaries can also be induced in a variety of animal models through estrogen administration (Convery and Brawer, 1991); however, there are no generally acceptable animal models of the pathophysiology of PCOS. Neither are there data on trends in the incidence of PCOS in women.

5.1.8.3 Shortened lactation. Lactation is a critically important process that provides nutrition to the newborn. There are two physiological processes of milk secretion, with let down as well as milk ejection, controlled by a cascade of neurotransmitters from the anterior and posterior pituitary gland. The theoretical problems that endocrine disruption may cause are 1) the transfer of maternal endocrine disruptors to the infant, with subsequent adverse health effects, and 2) induced abnormalities in the production of milk or its release.

Lactation in women can be affected by pharmacological doses of combined estrogen:progestagen oral contraceptives and they are contraindicated until weaning has taken place. A study in a region of northern Mexico, where DDT has been heavily used in agriculture and high human breast milk and fat levels of DDE have been recorded, has shown that the concentration of DDE in the breast milk correlated with duration of lactation. In women with breast milk concentrations of *p,p′*-DDE ≥ 12.5 ppm, the median duration of lactation was 3.0 months, compared with 7.5 months in those with the lowest concentrations of <2.5 ppm (Gladen and Rogan, 1995). Differences remained significant after exclusion of women ceasing lactation for discernible external reasons. In rodents, lactation has been shown to be impaired by exposure to the pesticide atrazine through inhibition of prolactin (Kniewald et al., 1987; Cooper and Kavlock, 1997; Stoker et al., 1999a, 1999b, 1999c). However, no conclusions can be drawn from these sparse human and animal data.

5.1.9 Conclusions and Recommendations on Reproduction

The major limiting factor in drawing any conclusions about human reproductive health effects and putative links to EDCs is the absence of exposure data. Exposure data are very limited, if available at all, and in many studies exposure has only been inferred and not actually measured. Another major problem common to many of the human studies is that sample sizes are often too small to permit detection of an effect, even if one was present. Thus, the currently available human data are inadequate to support a conclusion that human reproductive health has been adversely affected by exposure to EDCs. Similarly, although there is evidence for geographical differences and temporal trends in some aspects of human reproduction, there has been no systematic attempt to look for evidence that the mechanisms behind these changes involve endocrine pathways.

Despite these drawbacks, the biological plausibility of possible damage to human reproduction from exposure to EDCs seems strong when viewed against 1) the background of known influences of endogenous and exogenous hormones on many of the processes involved, and 2) the evidence of adverse reproductive outcomes in from wildlife and laboratory animals exposed to EDCs. The biological plausibility and the striking changes in human reproductive health trends in some areas, for some outcomes, are sufficient to warrant concern and make this area a research priority.

Data on effects of chemicals on female reproductive health are particularly sparse both in the human and experimental literature. Concerns about females derive mainly from biological knowledge about the influence of sex hormones on development and adult reproductive function, rather than from studies on environmental chemicals. Evaluation of possible links to EDCs could be pursued by exploiting identified temporal trends and geographical differences in the sex ratio, whereas the frequency of endometriosis in the general population offers opportunities for human studies requiring fewer numbers than might be required for other end points. Time to pregnancy can be used as an apical tool to broadly examine the possible influence of environmental chemicals on both male and female reproductive function.

With respect to effects on males, several meta-analyses and single retrospective studies suggest that there could be a decline in sperm quality in some regions over time, whereas other studies have not found a decline. Thus, the evidence to date certainly does not support the hypothesis that there is a worldwide decline. Because of the intra- and interindividual variability of human semen characteristics, the heterogeneity of study populations, lack of information defining the study populations, sample size limitations of many studies, and uncertainties of the quality and standardization of most published studies, the possibility of temporal declines in sperm production and fertility in some regions remains open. The geographical variation of sperm count and concentration is a more real phenomenon. The differences observed between large similar populations of healthy men cannot readily be explained by methodological or confounding factors alone. It could be related to environmental factors but also to genetic factors. For the future, prospective studies in well-defined populations and various categories of the population in the same place are needed, in order to determine if changes in testis function are real and extend to the general population. Studies of men exposed to known EDCs are also very sparse. Longitudinal and case–control studies of men exposed to suspected compounds should also be informative, provided other factors that may interfere with male reproductive health are taken into account.

The prevalence of male reproductive tract disorders, such as cryptorchidism and hypospadias, also needs more careful study, particularly because they may be linked to testicular cancer that shows marked temporal increases in many countries. Experimental data in animals suggest that in both the adult and the developing organism, the prostate may be readily influenced by endocrine-active chemicals. To date, there is no human information on prostate changes in relation to early or adult exposures and the difficulties of investigating aspects such as growth and subtle cellular changes in the prostate should not be underestimated.

Because temporal trends in human sperm quality have fueled much of the debate about EDCs and could have a considerable impact within large populations on the proportion of men who are subfertile or infertile, this area warrants high priority for further research.

5.2 Neurobehavior

5.2.1 Introduction

As noted in Chapter 3, the nervous system plays an integrative role along with the endocrine and immune systems in orchestrating important physiologic functions of the body. These integrative functions are critical for normal development, cognitive functions, and behavior. A number of environmental chemicals (including potential EDCs) have been shown to cause neurotoxic effects (IPCS, 1986; NRC, 1992; IPCS, 2001b). A variety of adverse health effects have been observed ranging from motor impairment and memory loss to subtle behavioral changes (Spencer and Schaumburg, 2000). Of particular concern are the potential effects of exposures on the developing nervous system, because both the nature and adversity of the outcome may depend on the time window during which chemical exposure occurs and may result in irreversible neurobehavioral changes later in life (Tilson, 1998).

The complexity of the nervous system as well as its integrative nature offers multiple potential target sites that may be disrupted through a variety of mechanisms, including endocrine-disrupting mechanisms. Chemical induced effects may be direct, that is, due to an agent or its metabolites acting directly on sites in the nervous system; or indirect, that is, due to agents or metabolites that produce their effects primarily by interacting with sites outside the nervous system. Unless the mechanisms of action are known, it is often difficult to distinguish between direct and indirect effects, even for chemicals that have the known potential to influence hormone action (Chapter 3, section 3.15). Typically, neurobehavioral functions are not directly affected by chemicals but result from chemical-induced morphological and/or functional alterations in a variety of neuroendocrine pathways. This section reviews the extent to which neurobehavioral changes following exposure to neurotoxic chemicals may be related to mechanisms of endocrine disruption. Only neurotoxic chemicals for which effects on endocrine systems may have relevance for neurobehavioral alterations are considered here. These include organochlorine pesticides such as DDT and/or its metabolite DDE, chlordecone (kepone), chlordane, some fungicides (methoxychlor, fenarimol), and, in the PHAH category, the polychlorinated dibenzodioxins (PCDD), polychlorinated/brominated biphenyls (PCB, PBB), and dibenzofurans (PCDFs).

5.2.2 Human Data

5.2.2.1 Developmental neurobehavior.

5.2.2.1.1 PHAHs. The epidemiological literature on neurodevelopmental effects of PCBs published up to 1995 has been reviewed by Schantz (1996). Apart from two mass poisoning events in Japan in 1968 (Yusho) and in Taiwan in 1979 (Yu-Cheng) in which 1,000–2,000 adults were accidentally exposed to high levels of PCBs (and other PHAHs) through contaminated rice oil, there are now four additional cohort studies in which measured PCB concentrations at environmental background levels in relevant body fluids have been related to developmental and neurobehavioral outcomes.

Despite the high levels of exposure (Yu-Cheng mothers: median serum PCB concentrations were 26.8 ng/ml according to Guo et al., 1995a) and the possible contribution of other PHAHs (e.g., PCDFs), both the Yusho and Yu-Cheng episodes, as described by Schantz (1996), provide sufficient neurodevelopmental information for further analysis. In the Yusho incident, apart from the predominant dermal effects (acneform lesions, brown skin pigmentation), other frequent symptoms in affected adults were of central and peripheral nervous system origin and included headache, loss of memory, and hypoesthesia or neuralgia of the limbs. Women who were pregnant when poisoned gave birth to small babies with dark brown skin and other abnormalities. In a subset of children who were followed for several years, persistent growth retardation, movement disorders, generalized slowness, and substantially IQ deficit (average IQ was around 70) were found. In the Yu-Cheng incident the overall picture was similar to the Yusho episode, although the babies born to mothers exposed during pregnancy were followed more carefully over a longer period of time and compared with carefully matched controls (Rogan and Gladen, 1992). A small but systematic lowering of IQ (by one-third of a standard deviation), prolonged p_{300} latencies, and smaller p_{300} amplitudes (p_{300} is a late positive-going component of the event-related brain potential, contingent upon decision/cognitive processes, with latencies of around 300 msec after stimulus onset), as well as evidence of behavioral disorders and increased activity, were reported for the cases relative to the matched controls. However, there was no correlation between the degree of deficit and the PCB levels of the mothers (Schantz, 1996). Because pregnant mothers were advised by their doctors not to breast-feed, effective PCB exposure was most likely prenatal.

Four additional cohort studies, in which measures of internal dose were related to neurobehavioral outcome at different postnatal ages, are currently available; two U.S. studies conducted in Michigan and North Carolina, and two European studies (the Dutch breast milk and the European PCB studies). In the Michigan study, healthy mother–infant pairs were recruited from families with different consumptions of Lake Michigan fish; the other three studies are general population studies. All of them are characterized by background PCB levels measured in different matrices, namely, maternal serum, umbilical cord serum, and/or maternal milk collected shortly after birth. In the Michigan study (Jacobson et al., 1990), out of over 8,000 mothers who had given birth to a healthy child, a total of 313 were recruited for the study who had reported different quantities of fish consumption over the past 6 years and a control group with no self-reported consumption of Lake Michigan fish. An effort was made to measure PCBs in maternal and umbilical cord serum as well as in maternal milk of breast-feeding mothers. PCB-values were available for only about 30% of the cord sera. Mean cord serum levels were 3 ± 2 ng/ml; milk levels were 841 ± 386 ng/g fat (Jacobson and Jacobson, 1996). Neurobehavioral testing took place at several ages between birth and 11 years of age. The overall outcome was as follows (Schantz, 1996): (1) fish consumption but not PCB cord/milk was negatively correlated with motor development, hyporeflexia, and lability at birth; (2) neither fish consumption nor PCB cord/milk was associated with the mental or psychomotor development at 5 months; (3) visual recognition memory was negatively related to PCB cord but not PCB milk at 7 months; (4) at 4 years of age, higher PCB levels in both umbilical cord serum and maternal milk were associated with poorer performance on the McCarthy verbal and numerical memory subtests; and (5) the full-scale and verbal IQ exhibited a negative association with a composite exposure index constructed from PCB/maternal/cord/milk at 11 years (Jacobson and Jacobson, 1996). In summary, postnatal neurobehavioral development appears to be negatively related to *in utero* but not to postnatal PCB exposure.

In the North Carolina study (Gladen et al., 1988), 880 mother–infant pairs were recruited from the general population with more than 700 available for follow-up until 5 years of age. Because PCBs were not detectable in cord serum, they were measured in maternal milk of breast-feeding mothers shortly after birth as well as on later occasions up to 12 months (median at birth, 1.770 ng/g fat;

maximum, 16,000 ng/g fat). Neurobehavioral development of the children was measured at regular 6/12-month intervals after birth up to 5 years of age. Anthropometric measures (height, weight, head circumference) were taken as well. Hyporeflexia and hypotonicity at birth and delayed motor development up to 24 months were associated with prenatal PCB body burden of the mothers indexed by PCBs in early milk samples; no such association was observed beyond 24 months of age. Cognitive development was not affected at any postnatal age. Again, as in the Michigan study, neurodevelopmental delay was taken to be related to *in utero* rather than to postnatal PCB exposure.

In the Dutch breast milk study (Huisman et al., 1995; Koopman-Esseboom et al., 1996), 200 healthy mother–infant pairs were recruited in each of the university hospitals of Groningen and Rotterdam, respectively. Half of the mothers were breast-feeding, the other half formula feeding. Four PCB congeners were measured in maternal and cord plasma and additional PCBs as well as a number of dioxins in early breast milk samples. PCB levels (sum of 118, 138, 153, 180) were as follows: cord plasma, median: 0.43 ng/ml; maternal plasma, median: 2.2 ng/ml; milk, median: 366 ng/g fat. Neurological status and psychomotor and mental development were assessed at 2 weeks, and at 3, 7, and 18 months of age. Additionally, at 7 months, visual recognition memory was measured. Neurological status (hypotonia but not hyporeflexia) exhibited negative associations with PCBs in maternal plasma but not with PCB/dioxins in milk at 2 weeks and 7 months. Psychomotor, but not mental development, was delayed at 3 and 7 months in relation to PCBs in maternal plasma but not cord plasma or milk. At 18 months, the overall neurological status as well as fluency of movements exhibited negative association with PCBs in cord plasma (Huisman et al., 1995). No impairment of visual recognition memory was found to be associated with neonatal PCBs at 3 and 7 months. A follow-up of these two cohorts at 42 months of age was done within the European PCB study described below.

In the European PCB study, in addition to the two Dutch cohorts described above, two additional cohorts of about 170 healthy mother–infant pairs were formed, a Danish cohort from the Faroe Islands and a German cohort from Duesseldorf. The two newly formed cohorts were studied at 2 weeks, 7, and 18 months for neurodevelopment and psychomotor/mental development; the two Dutch cohorts were reassessed for neurodevelopment, language development, and cognitive development at 42 months of age. The common denominator for neonatal PHAH exposure was PCBs in cord and/or maternal plasma as well as in early breast milk samples. PCB levels were as follows (for the Dutch cohorts, see above): for the Faroe Islands cohort, no cord plasma values; milk, 874 ng/g fat (median); for the Duesseldorf cohort, cord plasma, 0.41 ng/ml (median); milk, 405 ng/g fat (median). Results from these studies suggest that (1) mental and motor development between 7 and 42 months of age is negatively associated with PCBs in breast milk but not with cord plasma PCBs, and the degree of association becomes formally significant from 30 months onward (Walkowiak et al., 2001), (2) visual recognition memory does not relate to neonatal PCB at 7 months of age (Winneke et al., 1998), (3) both cognitive development and language development exhibit negative association with PCBs in maternal plasma but not in cord plasma at age 42 months (Patandin et al., 1999), and (4) neurodevelopmental effects are not associated with neonatal PCBs at 42 months of age (Lanting et al., 1998).

In summary, PCBs have been reported to have a negative impact on neurobehavioral development. Delays in postnatal psychomotor or neurological as well as cognitive development have been found to be associated with neonatal PCB exposure (marker congeners 118, 138, 153, 180), although the implicated PCB matrix (maternal vs. cord plasma or early breast milk) differs between studies. The degree of persistence of developmental delay is controversial and the mechanistic basis of such effects is unclear; a hypothyroid mechanism of action (perhaps related to endocrine disruption) of PCBs is discussed below.

5.2.2.1.2 Possible role of thyroid dysfunction. Because of the organizational role of thyroid hormones for development in general and for brain development in particular (Chapter 3), hypothyroid action of PHAHs during development could be a mechanism by which neurobehavioral dysfunction is mediated (Porterfield, 1994). Hypothyroid alterations in association with neonatal PCBs/PHAHs at background environmental levels of exposure have been reported fairly consistently in developmental studies in infants. Within the Dutch breast milk study, negative associations between T_4, T_3 in infant cord plasma, and PCB/PCDD TEQ in maternal (but not cord) plasma, and a positive association with plasma TSH at 2 weeks and 3 months were found (Koopman-Esseboom et al., 1995). Exposure ranges in terms of toxic equivalence factors (TEQ) in human milk were 30.85–154.21 pg TEQ/g fat for total PCB/dioxins. In another Dutch study, TSH elevation together with (unexpected) elevated T_4 was found in association with exposure to PCDF/PCDD in young children; two groups designated "low" (8.7–28 ng dioxin TEQ/kg milk fat) or "high" (29.2–62.7 ng TEQ/kg fat) were compared (Pluim et al., 1993). Also, in a Japanese study, T_4 levels in serum of babies at 1 year of age exhibited negative association with TCDD TEQ (range, 15.2–48.5 ppt, fat basis) measured in maternal milk taken 3 months after birth; other thyroid function parameters were not affected (Nagayama et al., 1997). In all of these studies thyroid hormone levels were in the normal range. As yet it is unclear if such subclinical alterations can be responsible for later neurobehavioral deficit or delay. In one isolated study (Koopman-Esseboom, 1995), no differences were found between neurologically normal (n = 394) and a few abnormal children (n = 23) in terms of thyroid function parameters. However, if exposure during early prenatal life is important, these studies do not give information on thyroid status in the relevant time period.

5.2.2.2 Adult nervous system.

5.2.2.2.1 PHAHs: neurobehavior and thyroid effects. Few studies have examined at neurobehavioral and/or thyroid PHAH effects in adults in relation to occupational or high environmental exposure. Patients from the Yu-Cheng episode exhibited lower nerve conduction velocities and various neurological symptoms, including impaired memory and dullness; PCB levels were 39.3 ± 16.6 ng/ml in blood (Chen et al., 1985). Long-lasting peripheral neuropathy and encephalopathy was found in three cases of PCB-exposed capacitor repair workers exposed for between 4 and 20 years; no data on internal exposure are available (Altenkirch et al., 1996). Firemen exposed to high levels of PCBs mainly judged from the external exposure situation (internal PCB exposure was given in terms of Arochlor 1248 as a median serum PCB level of 6.0 µg/g fat) differed from matched controls in different psychological tests (e.g., short-term memory, visual-motor performance, reaction time); no correlation was found between degree of impairment and individual PCB serum levels (Kilburn et al., 1989). No thyroid effects but a positive correlation between adipose tissue PCBs and 17-hydroxycorticosteroid excretion was found in transformer repair workers (Emmett et al., 1988). Increased thyroid volume but no change in other thyroid function parameters was found in former

employees of a PCB-producing plant or in adolescents living in the polluted neighborhood relative to controls (Langer et al., 1998).

5.2.2.3 Sex hormones and gender-dependent behavior. The role of gonadal hormones during development in affecting behavioral sex differences in humans has recently been reviewed in a comprehensive and hypothesis-guided manner (Collaer and Hines, 1995). In this review, clinical syndromes of hormonal imbalances as well as intentional prenatal exposure to DES and progestins (given to manage at-risk pregnancies) are covered. Girls born to DES-treated mothers appear to be masculinized or defeminized in terms of sexual orientation and language laterality, but not in other respects (core sexual identity, childhood play, cognition). Boys born to DES mothers display less characteristic behavioral changes and evidence on alterations of male behavior. Prenatal exposure of girls to androgen-based progestins was found to be associated with male behavior (e.g., increased tomboyism, higher preference for male-typical toys and male playmates), and more aggression. As for boys, effects, if any, are more subtle, for example, slightly more physical aggression and a tendency to reduced male-typical play. Effects following prenatal exposure to progesterone-based progestins are weak and inconsistent. This information is relevant in the present context, because it can serve as a frame of reference for what may or may not be expected from exposure to environmental low-dose exposure to EDCs in terms of interaction with sex hormones and resulting behavioral alterations. It appears that prenatal exposure to synthetic estrogens (such as DES) or androgen-based progesterone has stronger and more characteristic sexual behavioral effects in girls than in boys.

5.2.2.3.1 PHAHs. Experimentally, interaction of PHAHs with sex steroids and resulting behavioral disruption has clearly been demonstrated in rodents but human data are rare. Some estrogenic-like action of PHAHs has recently been reported (Lanting, 1999). In that study higher volumes of human milk and fat content in breast-feeding mothers was positively associated with maternal PCB burden. Shorter penis length has been reported in connection with the Yu-Cheng incident. Whether or not the behavioral abnormalities in children from this cohort may be interpreted as indicating androgenic or antiestrogenic action of prenatal PHAH exposure needs closer attention.

A gender-dependent behavioral PCB effect was observed in children from the Yu-Cheng cohort. In nonverbal tests for general intelligence based on the solving of maze problems of increasing task difficulty, boys but not girls at 6–9 years of age from the exposed group scored significantly lower than matched controls (Guo et al., 1995b). This suggests of a disturbance of sex hormones due to prenatal PCB/PCDF exposure. In following up the North Carolina cohort until puberty, no evidence of effect, in terms of onset or time course and behavioral manifestations of puberty, was found (Gladen et al., 1996).

5.2.2.3.2 Pesticides. It is known that occupational exposure to pesticides may cause infertility and sterility through hormonal imbalance in males (Strohmer et al., 1993; Straube et al., 1999). Little is known, however, of the possible links, if any, between the known neurotoxicity of many pesticides and any underlying hormonal influence.

Gladen et al. (1988) reported that both PCB and DDE concentrations in maternal milk (median value of 1.77 µg/g fat, and individual values ranging up to 16 µg/g) exhibited an association with abnormal reflexes and hyporeflexia at birth, whereas hypotonicity was only reported for PCBs. It is uncertain if this can be considered a true DDE effect or if it represents a spurious correlation due to the colinearity of PCBs and DDE.

5.2.3 Animal Data

Epidemiological studies on neurobehavioral effects of endocrine disruptors can be corroborated by results of experimental investigations. The focus of this review is on the spectrum of neurobehavioral effects of endocrine-active xenobiotics and sensitive exposure periods. It should be noted, however, that there are many substances for which endocrine effects have been described but that have not yet been examined for neurobehavioral effects.

5.2.3.1 Developmental exposure.

5.2.3.1.1 Sex-dependent neurobehavioral effects. Sex-dependent neurobehavioral effects are responses that are differentially expressed in both sexes but that are not directly related to sexual functions and reproduction. Table 5.6 summarizes studies on such effects following developmental exposure.

Table 5.6 - Neurobehavioral Effects of Developmental Exposure to Endocrine-Effective Compounds in Rats: Sex-Dependent Effects

Compound	Dose and Regimen	Effect	LOAEL	NOAEL	Reference
Chlordane	0.1, 0.5, or 5 mg/kg; GD 4 to PND 80	Improvement of spatial learning (Cincinnati maze) in female offspring	0.1 mg/kg	–	Cassidy et al., 1994
Chlordecone	1 mg/pup on PND 4	Decreased auditory startle responses after a challenge with harmine in adult male offspring, increase in adult female offspring	1 mg/pup	–	Mactutus and Tilson, 1985
Nicotine	0.25 mg/kg/hr by osmotic minipumps; GD 12–20	Elevated sweet preference in adult male offspring	0.25 mg/kg	–	Lichtensteiger and Schlumpf, 1985
PCB 28	8 or 32 mg/kg PO; GD 10–16	Deficit in delayed spatial alternation in female offspring	32 mg/kg	8 mg/kg	Schantz et al., 1995
PCB 118	4 or 16 mg/kg PO; GD 10–16	Deficit in delayed spatial alternation in female offspring	16 mg/kg	4 mg/kg	Schantz et al., 1995
PCB 153	16 or 64 mg/kg PO; GD 10–16	Deficit in delayed spatial alternation in female offspring	64 mg/kg	16 mg/kg	Schantz et al., 1995
PCB 77	1.5 mg/kg SC; GD 7–18	Reduced b-wave amplitudes in the electroretinogram of female offspring	1.5 mg/kg	–	Kremer et al., 1999
PCBs*	40 mg/kg diet, equivalent to 4 mg/kg bw/day, for 50 days before mating until PND 0	Decreased activity of aromatase in the HPOA of male pups at birth; elevated sweet preference in adult male offspring; no pronounced effects of an equal dose of the technical mixture Aroclor 1254 on these end points	4 mg/kg	–	Hany et al., 1999

*Reconstituted mixture (breast milk pattern). PO, per os; SC, subcutaneous injection; bw, body weight.

The elevated sweet preference in male offspring after exposure to nicotine or the reconstituted PCB mixture suggests a behavioral feminization that, for the PCBs, is most likely due to the inhibition of aromatase activity (Chapter 3). Impairments of delayed spatial alternation seen after exposure to single *ortho*-chlorinated PCB congeners suggest changes in the prefrontal cortex that receives a strong dopaminergic projection from the ventral tegmentum. Because dopamine release was shown to depend on estrogen level, this may be the cause for the occurrence of deficits only in females. However, estrogen level and cyclicity were not studied in this experiment (Schantz et al., 1995). The chlordane-induced improvement of spatial learning in females may indicate a masculinization because females perform better on this type of behavior in diestrus when estrogen levels are low. Reasons for sex-dependent changes on auditory startle with chlordecone (Mactutus and Tilson, 1985) and on the electroretinogram with coplanar PCB 77 (Kremer et al., 1999) are not yet known.

5.2.3.1.2 Sexual differentiation and gender-dependent behavior. Effects of endocrine-active compounds on developmental neuronal processes directly related to sexual behavior and reproduction are summarized in Table 5.7. Increased levels of mounting were observed in ovariectomized and testosterone-primed female offspring after chlordecone treatment (LOAEL, 0.5 mg/pup; NOAEL, 0.25 mg/pup; Gray, 1982).

The studies also indicate a demasculinizing effect of TCDD by impairment of malelike sexual behaviors (Mably et al., 1992). In addition, TCDD causes a feminization in males after castration and priming with ovarian steroids. However, some of these effects were expressed less severely in another study of TCDD-induced effects (Gray et al., 1995a, 1995b).

5.2.3.2 Postweaning exposure. Results of neurobehavioral studies devoted to effects on sex-dependent and sexual behavior following exposure to endocrine-active compounds are given in Tables 5.8 and 5.9. In addition to studies in rats, effects of methoxychlor on sexual behavior were also detected in hamsters after 2 weeks of daily exposure to 200 mg/kg orally. Treated ovariectomized females exhibited increased lordosis behavior after priming with progesterone (Gray et al., 1988). Both sex-dependent and sexual behaviors indicate estrogenic effects of methoxychlor. The same holds true for bisphenol A, whereas fenarimol results in demasculinization of males, as indicated by impairment of mounting behavior together with signs of decreased numbers of E_2 receptors in the brain. These effects are most likely due to a developmental inhibition of aromatase activity in the brain (Chapter 3).

5.2.4 Thyroid Hormones

Because of the established role of thyroid hormones in neural development (e.g., neurogenesis, migration of cells in the central nervous system, and cell differentiation), endocrine-active compounds that are assumed to exert neurobehavioral effects by mediation of thyroid hormone–dependent processes have been extensively studied in developing animals. The results are summarized in Table 5.10. In contrast to peripheral type I thyroxine 5′-deiodinase, there is an increase in activity of the type II deiodinase in the central nervous system in response to decreases of circulating T_4 to counteract a decrease in conversion of T_4 to T_3 in the brain. Such activity increases were detected after developmental exposure to single PCB congeners or a technical mixture (Morse et al., 1993, 1996). Another neurobehavioral effect of PCB, for which a mediation by action on thyroid hormones has been demonstrated, is the elevation of hearing thresholds in the low-frequency range; replacement of T_4 during development partially reversed the effects of PCB exposure (Goldey and Crofton, 1998). Although less clear, the same may be true for effects on choline acetyltransferase (Juarez de Ku et al., 1994). For influences on dopaminergic/serotonergic interaction in drug discrimination learning, a similarity of PCB-induced effects and effects by a thyrostatic compound has been shown, but a reversal by T_4 replacement has not yet been demonstrated (Lilienthal et al., 1997).

Table 5.7 - Neurobehavioral Effects of Developmental Exposure to Endocrine-Effective Compounds in Rats: Sexual Differentiation and Behavior

Compound	Dose and Regimen	Effect	LOAEL	NOAEL	Reference
Chlordane	0.1, 0.5, or 5 mg/kg, GD 4 to PND 80	Decreased intromission latencies, elevated number of intromissions prior to ejaculation and elevated number of total intromissions in male offspring	0.1 mg/kg	–	Cassidy et al., 1994
Fenarimol	350 mg/kg diet, equivalent to 35 mg/kg bw, GD 0 to PND 5	Decreased concentrations of nuclear ERs in HPOA of male neonates; reduced concentrations of E_2 and estrone in HPOA of female neonates	35 mg/kg	–	Hirsch et al., 1987a, 1987b
TCDD	64, 160, 400, or 1,000 ng/kg bw PO on GD 15	Demasculinization: prolonged mount, intromission, and ejaculation latencies in male offspring. Feminization: elevated lordosis quotient and lordosis scores in castrated male offspring primed with ovarian steroids	64 ng/kg 160 ng/kg	– 64 ng/kg	Mably et al., 1992
TCDD	700 ng/kg PO on GD 15	Replication of the results by Mably et al. 1992; effects not due to changes in numbers of ERs or in volumes of sexually dimorphic brain nuclei	700 ng/kg	–	Bjerke et al., 1994
TCDD	1,000 ng/kg PO on GD 15	According to cross-fostering, feminized sexual behavior in male offspring due to lactational exposure, effects on demasculinization inconclusive	1,000 ng/kg	–	Bjerke and Peterson, 1994
TCDD	1,000 ng/kg SC, GD 8 or 15	Male sexual behavior less affected or not affected at all by TCDD in contrast to studies by Mably et al. (1992) and Bjerke et al. (1994)	1,000 ng/kg	–	Gray et al., 1995b
TCDD	1,000 ng/kg on GD 15	Vaginal thread in female Long-Evans offspring and difficulties in mating resulting in increased number of mounts without intromissions and elevated ejaculation latencies in unexposed stud males	1,000 ng/kg	–	Gray and Ostby, 1995
TCDD	1,000 ng/kg PO on GD 15	Level of ER mRNA increased in the hypothalamus, uterus, and ovaries and decreased in the pituitary; DNA binding was elevated in the uterus, reduced in the hypothalamus and not altered in the ovaries of peripubertal female offspring	1,000 ng/kg	–	Chaffin et al., 1996
Vinclozolin	200 mg/kg, GD 14 to PND 3	Impaired intromissions and ejaculations in male offspring	200 mg/kg	–	Gray et al., 1994

bw, body weight; PO, per os; SC, subcutaneous injection.

Table 5.8 - Neurobehavioral Effects of Postweaning Exposure to Endocrine-Effective Compounds in Rats: Sex-Dependent Behavior

Compound	Dose and Regimen	Effect	LOAEL	NOAEL	Reference
Methoxychlor	400 mg/kg PO on PND 22	Prior to ovx elevated running wheel activity in treated females and no reduction after ovx in contrast to ovx controls	400 mg/kg	–	Gray et al., 1988
Methoxychlor	200 mg/kg PO from PND 104 throughout testing	After ovx elevated running wheel activity inexposed females that was antagonized by progesterone	200 mg/kg	–	Gray et al., 1988

PO, per os; ovx, ovariectomy.

Table 5.9 - Neurobehavioral Effects of Postweaning Exposure to Endocrine-Effective Compounds in Rats: Sexual Behavior

Compound	Dose and Regimen	Effect	LOAEL	NOAEL	Reference
Bisphenol A	200 mg/kg SC daily for 3 days	Lordosis quotient = 1 in ovx females	200 mg/kg	–	Gray and Ostby, 1998
Fenarimol	4, 8, 17, 35, or 70 mg/kg, PND 21–270	Decreases in number of mounts and increased mount latencies in males	4 mg/kg	–	Gray et al., 1991
Fenarimol 3	50 mg/kg diet, equivalent to 35 mg/kg bw, 7 days	Slightly (not significantly) elevated concentrations of nuclear E_2 receptors in HPOA and pituitary of treated ovx females	35 mg/kg	–	Hirsch et al., 1987a, 1987b
Fenarimol	350 mg/kg diet, equivalent to 35 mg/kg bw, 2 weeks	Nonsignificant reductions by 54% in concentrations of nuclear E_2 receptors in HPOA and pituitary of treated adult males	35 mg/kg	–	Hirsch et al., 1987a, 1987b
Methoxychlor	400 mg/kg PO, PND 22 to adulthood	Lordosis quotient (no. lordosis/no. mounts) = 1 in ovx exposed females after priming with progesterone, while = 0 in primed ovx controls	400 mg/kg	–	Gray and Ostby, 1998
Methoxychlor	200 mg/kg PO, PND 104 throughout testing	Lordosis quotient = 1 in ovx females	200 mg/kg	–	Gray et al., 1988
Methoxychlor	50 or 200 mg/kg PO, PND 21 to adulthood	Increased number of mounts and decreased sperm counts in males	50 mg/kg	–	Gray and Ostby, 1998
Polychlorinated biphenyls	10 mg/kg, daily for 30 days as adults	Reduced percentage of females with sperm in vaginal smear (reduced receptivity?)	10 mg/kg	–	Brezner et al., 1984

bw, body weight; ovx, ovariectomized; PO, per os.

Table 5.10 - Neurobehavioral Effects of Developmental Exposure to Endocrine-Effective Compounds in Rats: Thyroid Hormone–Mediated Effects

Compound	Dose and Regimen	Effect	LOAEL	NOAEL	Reference
PCB 126	250 or 1,000 ng/kg PO, 35 days before mating until PND 21	Elevated hearing thresholds in low-frequency range	250 ng/kg	–	Crofton and Rice, 1999
PCB 77	1 mg/kg SC, GD 7–18	Reduced blocking of apomorphine reaction by buspirone in exposed offspring resembling effects of developmental exposure to a thyrostatic compound	1 mg/kg	–	Lilienthal et al., 1997
PCB 169	0.2, 0.6, or 1.8 mg/kg PO on GD 1	Increased activity of type II T4 5´-deiodinase in whole brain of dams, fetuses (GD 20), and offspring on PND 7 and PND 21	1.8 mg/kg	0.6 mg/kg (no effect at 1.8 mg/kg on GD 20)	Morse et al., 1993
PCB 77 and PCB 169	PCB 77, 1 mg/kg PO, with PCB 169, 0.6 mg/kg, GD 2–18	Increased activity of type II thyroxine 5´-deiodinase in whole brain of fetuses (GD 20), reduced activity in female offspring on PND 21	1 mg/kg PCB 77 + 0.6 mg/kg PCB 169	–	Morse et al., 1993
PCBs, technical mixture (Aroclor 1254)	1, 4, or 8 mg/kg PO, GD 6 to PND 21	Reduced auditory startle amplitudes in offspring on PND 24; permanent elevated hearing thresholds in low-frequency range	4 mg/kg	1 mg/kg	Goldey et al., 1995
PCBs, technical mixture (Aroclor 1254)	1, 4, or 8 mg/kg PO, GD 6 to PND 21	Reduced amplitudes of auditory evoked responses in low-frequency range in offspring	4 mg/kg	1 mg/kg	Herr et al., 1996
PCBs, technical mixture (Aroclor 1254)	8 mg/kg PO, GD 6 to PND 21	Reduced auditory startle amplitudes in offspring on PND 23 and elevated amplitudes in adults; permanent elevated hearing thresholds in low- and high-frequency ranges; reversal of increases in low-, but not high-frequency thresholds by replacement of T_4, no reversal effects on startle amplitudes	8 mg/kg	–	Goldey and Crofton, 1998
PCBs, technical mixture (Aroclor 1254)	62.5, 125, or 250 mg/kg diet, equivalent to 6.25, 12.5, or 25 mg/kg bw, GD 0 to PND 21	Reduced activity of choline acetyltransferase in hippocampus and basal forebrain of exposed offspring, partial amelioration in hippocampus by T_4	6.25 mg/kg	–	Juarez de Ku et al., 1994
PCBs, technical mixture (Aroclor 1254)	5 or 25 mg/kg PO, GD 10–16	Increased activity of type II T_4 5´-deiodinase in forebrain of fetuses (GD 20), reduced activity in female offspring on PND 21	5 mg/kg	–	Morse et al., 1996

bw, body weight; PO, per os.

5.2.5 Conclusions and Recommendations on Neurobehavior

A number of neurobehavioral alterations have been reported to be associated with pre-/neonatal exposure to PHAHs (mainly PCB), although discrepancies exist in terms of the spectrum of effects. Although fairly consistent hypothyroid effects have been found in association with PHAHs in the pre-/neonatal period, a causal role in neurobehavioral dysfunction cannot be deduced from available human data; this is also true for interactions of PHAHs with sex steroids. Biological plausibility is provided by experimental work in animals on some potential endocrine disruptors that indicates exposure-related effects on sex-dependent and sexual behaviors, mediated via sex steroids. In human epidemiological work, measures on these types of behaviors have rarely been included. An exception is a study in Yu-Cheng children, reporting impairments in a special spatially structured intelligence test only in boys. Experimental studies also indicate a thyroid hormone–mediated influence on certain neurobehavioral endpoints, which can be disrupted if exposure occurs during critical periods of development.

Investigations of endocrine disruptors and effects on gonadal steroids and, to a lesser extent, on thyroid hormones have received most of the focus. Possible effects on other hormones due to impaired synthesis, enhanced metabolism, or changes at their targets should also be examined. Gonadal steroids have been implicated in various processes related to neural plasticity, including development, regeneration after injuries, and aging as well as protection against various diseases of the nervous system, neurotoxicants, oxidative stress, and other noxious influences. Endocrine disruptors may alter hormonal actions in all these processes, rendering the nervous system more susceptible to harmful events. Influences on neural plasticity may also impair the ability of adult organisms to adapt to environmental changes. In addition, it is known that the nervous system itself produces the so-called neurosteroids such as pregnenolone, dehydroepiandrosterone, progesterone, and its metabolites, independently of peripheral synthesis. There is no information to date concerning the effect of endocrine-active substances on this spectrum of steroid-mediated effects.

5.3 Immune System

5.3.1 Introduction

5.3.1.1 Outline of structure and function of the immune system. The major function of the immune system is defense against infectious agents and certain neoplastic cells. Various cell types and their soluble mediators execute the function of the system in a finely tuned manner. The host defense can be roughly divided into nonspecific or innate resistance and specific or acquired immunity mediated by lymphocytes (IPCS, 1996).

Components of the immune system are present throughout the body. The lymphocyte compartment is found within lymphoid organs that comprise the bone marrow and thymus, classified as primary or central lymphoid organs, and the secondary or peripheral lymphoid organs that include lymph nodes, spleen, and lymphoid tissue along secretory surfaces, the so-called mucosa-associated lymphoid tissue. Phagocytic cells of the monocyte/macrophage lineage, called the mononuclear phagocyte system, occur in lymphoid organs and also at extranodal sites, such as Kupffer cells in the liver, alveolar macrophages in the lung, mesangial macrophages in the kidney, and glial cells in the brain. Polymorphonuclear leukocytes, which are present mainly in blood and bone marrow and accumulate at sites of inflammation, execute a first line of nonspecific protection.

After initial contact of the host with the pathogen, specific immune responses are induced. The hallmark of this second line of defense is specific recognition of determinants, so-called antigens or epitopes, of the pathogens by receptors on the cell surface of B and T lymphocytes. Following interaction with a specific antigen, the receptor-bearing cell is stimulated to produce a clone of progeny cells that are specific for the eliciting antigen. The specific immune responses help the nonspecific defense presented to the pathogens by stimulating the efficacy of the nonspecific responses. A fundamental characteristic of specific immunity is that memory develops. Secondary contact with the same antigen provokes a faster and more vigorous but well-regulated response.

Two arms of specific immunity are recognized: humoral immunity and cell-mediated or cellular immunity. In humoral immunity, B lymphocytes are stimulated following recognition of antigen by cell-surface receptors. Mature B cells (plasma cells) start the production of antigen-specific immunoglobulins that act as antibodies in serum or along mucosal surfaces. The cellular immunity is mediated by T lymphocytes. They recognize antigen if presented by antigen-presenting cells in the context of histocompatibility antigens. Hence, these cells have a restriction in addition to the antigen specificity. T cells function as helper cells for various (including humoral) immune responses, mediate recruitment of inflammatory cells, and, as cytotoxic T cells, can kill target cells after antigen-specific recognition (reviewed in detail by Schuurman et al., 1991; IPCS, 1996; Weigle, 1997).

5.3.1.2 Immunotoxic responses that could result from EDCs. Toxic responses may occur when the immune system acts as a passive target of chemical insults, leading to altered immune function. Toxicity may also arise when the immune system responds to the antigenic specificity of the chemical as part of a specific immune response, that is, hypersensitivity or allergy. Chemical-induced toxicity, in which the immune system is the target, can result in immunosuppression and potential disease susceptibility, manifested as an increased incidence of infectious disease and certain tumor diseases, as well as the exacerbation of allergic and autoimmune disease (Schuurman et al., 1991; IPCS, 1996, 1999). Although immunotoxicity may occur following exposure to certain EDCs, it is important to distinguish between direct effects on the immune system and effects that are secondary to endocrine disruption. To date, the majority of immunotoxic reactions to chemicals (both EDCs and non-EDCs) where the mechanism is known do not involve endocrine effects. Nevertheless, the immune system and the neuroendocrine system communicate and cooperate closely to maintain physiological homeostasis (Chapter 3, section 3.15). Clearly, there is the potential for chemicals to influence immune function adversely via endocrine mechanisms, and the few known examples of these are discussed later.

Increases in disease in humans, the ultimate outcome of immune dysfunction, are detectable endpoints but causality is difficult to attribute. Thus, for the detection and evaluation of direct immunotoxic effects of chemicals, reliance must be placed on experimental models. A wide array of methods are available to assess immune function. In evaluating the immunotoxicity of chemicals, regulatory authorities in many countries require a multidose 28-day toxicity study in rats. This first screen includes a general set of indicators of the specific and nonspecific immune system, such as are incorporated in OECD guideline 407 (OECD, 1995a). These indicators evaluate toxicity to T- and B-lymphoid cells in primary and secondary lymphoid tissue, as assessed by the weight and the histology of the lymphoid organs. Examination of mucosa-associated

lymphoid tissue is also of value, especially when exposure occurs at the mucosal locations, as is the case in feeding studies. If the screening reveals immunotoxic effects not judged to be secondary to other toxic effects of dhe chemical and the effects occur at a dose level relevant in relation to other toxic effects, second-tier studies are indicated. At the second-tier there is great variety of *in vivo*, *ex vivo*, and *in vitro* assays to assess cell-mediated and humoral immunity, macrophage function, natural killer cell activity, and host resistance in experimental infection models. As understanding of EDCs progresses, it may be necessary to incorporate other tests for immunocompetence into the tiered array.

Host resistance models can be very helpful for risk assessment because they are tools to elucidate the actual consequences of disturbances of immune function. Different host resistance models address different components of the immune system, based on the mechanism in that particular model. Consequently, no single host resistance model can be used as the only tool to evaluate the influence on immunocompetence of exposure to immunotoxic agents (Neumann, 1995; IPCS, 1996).

5.3.2 Human Data

Studies during the last two decades in man and in laboratory animals have clearly shown that the immune system is a target for many compounds, including drugs and chemicals of environmental concern. However, the number of compounds causing immune alterations in humans via a proven endocrine-disrupting mechanism is limited to a few. For DES such a mechanism is clear. For the PCBs, PCDFs, and PCDDs, the toxicity to the thymus, as for most other target organs, appears mediated through AhR binding, resulting in likely effects on thymic hormones. The immunotoxicity may therefore be considered to be mediated through an endocrine-disrupting mechanism. Premature introduction of progesterone has been shown to induce immunosuppression, but the mechanisms reported have been controversial (Siiteri et al., 1977; Schust et al., 1996). A chemical may also show endocrine-disrupting activity but not affect immune function, as reported in the adult rat after perinatal/juvenile exposure to methoxychlor (Chapin et al., 1997a). Thus, for the majority of the few immunotoxic compounds for which the mechanism is known, endocrine disruption is not usually involved.

5.3.2.1 DES. The immunological effects in man and experimental animals following DES exposure have been reviewed by Blair et al. (1992) and Golden et al. (1998). Limited data are available on long-term immune effects in man following *in utero* exposure to DES. Evidence for immunostimulation was reported from increased lymphoproliferative responses to the mitogens PHA and PWM in eight women with reproductive tract abnormalities and evidence of cervical and/or vaginal adenosis (Ways et al., 1987). Another small study of daughters with reproductive tract changes consistent with *in utero* DES exposure suggested possible altered function of natural killer cells (Ford et al., 1983).

Two large DES-exposed cohorts were studied to examine the potential immunological consequences of *in utero* exposure. These studies suggest an increase in the lifetime prevalence of possibly impaired immune function, that is, respiratory tract infections, asthma, arthritis, and lupus, in DES-exposed individuals compared with that observed in the general population (Wingard and Turiel, 1988), or in a control group of nonexposed women participating in the project (Noller et al., 1988). In a follow-up study, using two different groups of DES-exposed women, with an appropriate control group for each, no difference in the prevalence or serum titer of antibodies to five common virus diseases and six less common ones was observed. However, an increased prevalence of a relatively rare immunological hyperreactivity, rheumatic fever, subsequent to microbial infection (strep throat) was found in DES-exposed women (Blair et al., 1992). In a further study, sera of DES-exposed and nonexposed women were examined for the presence of factors associated with autoimmune diseases, and additionally, immunoglobulin levels were determined (Blair, 1992). The incidence of high antibody titers to red blood cell antigen was found to be higher in the DES-exposed females than in the controls and serum IgA values were significantly increased. Blair (1992) concluded that, in general, humans exposed prenatally to DES do not exhibit severe defects in basic immune function, but their propensity to develop autoimmune disease and other diseases associated with defects in immune regulation appears to be increased.

5.3.2.2 PCBs, PCDFs, and PCDDs. For reviews on the effects of PCBs, PCDFs and PCDDs on the immune system, see Birnbaum (1995), Vos et al. (1997/98), and Golden et al. (1998).

5.3.2.2.1 Accidental exposure. Epidemiologic studies on residents of Seveso, Italy, some of whom were exposed to relatively high levels of TCDD, revealed no abnormalities in serum immunoglobulin concentrations and mitogen responses of T and B cells (Reggiani, 1978). Other accidental exposure situations have revealed immunotoxic effects in humans. In a comprehensive examination of people exposed to TCDD-containing waste oils, sprayed for dust control on a dirt road at a mobile home park in Missouri, USA, in 1971, immune alterations were noted. The exposed group had a statistically significant increased frequency of anergy and relative anergy on DTH skin testing and nonstatistically significant, increased frequencies of abnormal T-cell subset test results, a T_4/T_8 ratio of less than 1.0, and an abnormality in the functional T-cell test results (Hoffman et al., 1986). This suggests that long-term TCDD exposure is associated with suppressed cell-mediated immunity, although the question is still open as to whether these alterations were endocrine mediated. In the follow-up of individuals for whom immunologic anergy was shown, none was still anergic. The two most likely explanations for this phenomenon postulated by the authors are the weak potency of the skin test applied in the first study or sensitization to the antigen by the first test (Evans et al., 1988). Another study on the same group seeking clinical correlates with TCDD burden displayed significant increases in both the percentage and absolute number of CD8 T cells and a nonsignificant decrease in the CD4/CD8 ratio. Lymphocyte phenotype analyses in 15 children born to mothers who resided in the mobile home park during and subsequent to pregnancy revealed significantly low frequencies of the T-helper-inducer (CD29/CD4) subset, elevated frequency of the CD8 subset and decreased frequencies of the light-chain-bearing B cells (CD19) (Smoger et al., 1993). Another study of those exposed to TCDD in Missouri suggests an association between TCDD exposure and the activity of the thymic epithelium, with significantly lower mean serum level of the thymic peptide, thymosin-a1 (Stehr-Green et al., 1989). These data support an endocrine-mediated etiology of the immunotoxicity.

Immune alterations have been observed in Taiwanese residents exposed to TCDD-related polyhalogenated aromatic hydrocarbons. Consumption of rice oil accidentally contaminated with PCDFs and PCBs caused acneform skin lesions, pigmentation of skin and nails, liver damage, and abnormal immune function (Yu-Cheng disease). Serum IgM and IgA concentrations and the percentage of

T-lymphocytes in the peripheral blood were decreased (Chang et al., 1981). Investigations using DTH responses showed suppression of cell-mediated immunity (Chang et al., 1982). Children born to female Yu-Cheng patients were also examined. The exposed children had higher incidences of bronchitis during the first 6 months after birth (Rogan et al., 1988) and otitis media at school age (Chao et al., 1997), compared with controls.

As noted above, a disease similar to Yu-Cheng poisoning in Taiwan occurred in Japan in 1968 following exposure to rice oil contaminated with PCBs and PCDFs, the so-called Yusho disease. Yusho patients frequently suffered from respiratory infections. Serum IgA and IgM levels considerably decreased during the 2 years following the onset of poisoning but returned to normal in most cases. Respiratory symptoms persisted for longer time periods (Shigematsu et al., 1978).

Immune alterations were also described in workers exposed to PCDFs and PCBs following a PCB accident in Finland. Investigations showed decrease numbers of T cells in peripheral blood 5 weeks after exposure, with recovery in most cases to normal values 7 weeks later. Lowered T-helper/T-suppressor cell ratios were also observed. During the 7 months after the accident, most of the exposed persons had at least one upper respiratory infection (Elo et al., 1985). A study in workers 17 years after accidental exposure to TCDD showed that antinuclear antibodies and immune complexes were detected significantly more frequently in the blood of TCDD-exposed workers in comparison to matched controls (Jennings et al., 1988).

5.3.2.2.2 Occupational exposure. Occupational studies have shown few immune changes. Veterans of Operation Ranch Hand, the U.S. Air Force unit that sprayed TCDD-containing herbicides in Vietnam from 1962 to 1971, showed no evidence of a consistent relationship between immune system alteration (DTH responses, lymphocyte subpopulations, serum immunoglobulins and autoantibodies) and TCDD exposure category (Michalek et al., 1999). Among workers involved in decontamination work of a chemical plant, with moderately increased body burdens of TCDD and other PCDDs and PCDFs, no relevant alterations in comparison with a control group in peripheral blood lymphocyte subpopulations (Neubert et al., 1993) and lymphoproliferative responses (Neubert et al., 1995). No effects were detected on lymphocyte subsets in the blood or T- and B-cell mitogen-induced lymphoproliferative responses in industrial workers exposed to high doses of TCDD for several years, 20 years prior to testing (Tonn et al., 1996). However, the TCDD-exposed subjects showed a reduced response to human lymphocyte antigen-allogeneic lymphocytes and interleukin 2–boosted proliferation. It was concluded that the long-term immunosuppressive effect on T-helper function is likely mediated by a reduced functionality of individual cells rather than by a reduction in absolute cell numbers in the peripheral blood.

5.3.2.2.3 General population exposure. The effects of breast-feeding versus bottle-feeding and pre- and postnatal exposure on immunological parameters was investigated in healthy infants (the Dutch PCB/dioxin study) from birth to 18 months of age (Weisglas-Kuperus et al., 1995). The total study group consisted of 207 healthy mother–infant pairs, of which 105 infants were breast-fed and 102 children were bottle-fed. Prenatal PCB exposure was estimated by the PCB sum (PCB congeners 118, 138, 153, and 180). Postnatal PCB/dioxin exposure was calculated as a product of the total TEQ level in human milk (17 dioxin and 8 dioxinlike PCB congeners) multiplied by the weeks of breast-feeding. There was no relationship between pre- and postnatal PCB/dioxin exposure and upper or lower respiratory tract symptoms or humoral antibody production. Total and dioxin TEQ levels in breast milk correlated significantly with increased T-cell subpopulations in the infants. Pre- and postnatal exposure to PCBs and dioxins was significantly associated with reduced monocyte and granulocyte counts at 3 months but not at 18 months of age. Additionally, significantly decreased B-cell markers were observed in the breast-fed group following posnatal exposure. In a follow-up study, to investigate whether these changes persisted into later childhood, in preschool children prenatal PCB exposure was associated with increased T-cell numbers, lower antibody levels to measles, and less shortness of breath with wheeze. Current body burden was associated with a higher prevalence of recurrent middle ear infection and chickenpox and a lower prevalence of allergic diseases (Weisglas-Kuperus et al., 1995, 2000).

5.3.3 Experimental and Animal Data

5.3.3.1 DES. As discussed in Chapter 3 (section 3.15), Luster et al. (1984) provided evidence that immunotoxicity of some estrogenic compounds, including DES, correlated for the most part with estrogenicity. Immunotoxicity of exogenous estrogens was mainly manifested as changes in the thymus and thymus-dependent immunity and occurred at pharmacological dose levels, raising the question whether immunotoxic effects can be expected at all with low exposures to weak estrogens. This is in contrast to TCDD, which is a potent immunotoxic compound at low exposure levels (section 5.4.3.2).

The mechanisms responsible for thymotoxicity appear to be mediated through a direct chemical interaction with thymocytes, as well as with nonlymphoid thymic epithelial cells, resulting in the release of soluble immunoregulatory factors by the epithelial cells, probably through binding to ERs or receptorlike structures. From studies performed in laboratory animals, Golden et al. (1998) concluded that they are generally consistent with effects reported in humans exposed *in utero.* Overall, a substantial amount of animal data demonstrate numerous immune alterations following *in utero* exposure to DES, including abnormal B-cell and T-cell responses and diminished natural killer cell activity. The relationship between these effects and neoplasia in rodents is unknown, as is the relevance of these findings to possible health consequences in humans. Most of the immune effects following *in utero* exposure persisted for the lifetime of the animal, and some even became more severe with age. Hence, continued surveillance of humans exposed *in utero* to DES for diseases related to immune dysregulation is warranted.

5.3.3.2 TCDD. Many studies have been performed to investigate the mechanism of TCDD-induced thymic atrophy (reviewed by Vos and Luster, 1989; De Waal et al., 1997). Some indirect mechanisms could be excluded, such as a contribution of stress hormones, because adrenalectomy or hypophysectomy did not influence the TCDD effect. Any role for growth hormone, reduced food intake, or zinc deficiency was also excluded. In mice it was established that the susceptibility to TCDD is genetically determined; susceptibility segregates with the locus encoding a cytosolic receptor protein mediating aryl hydrocarbon hydroxylase activity. This AhR has a high affinity for TCDD, and high levels of this receptor are found in the mouse thymus (of responsive strains) and in the rat thymus. As thymic atrophy is AhR mediated, this response has been used to develop toxic equivalent factors for TCDD congeners (Safe, 1990; Vos et al., 1997/98). In addition,

the splenic plaque-forming cell response to sheep red blood cells, a thymus-dependent humoral immune response that is also AhR mediated, has been used for this purpose.

The effect of TCDD on the thymus may be through an action on epithelial cells, although bone marrow prothymocytes have also been claimed to be targets for TCDD-induced thymus toxicity (De Waal et al., 1997). Thymus atrophy through an effect on epithelial cells is indicated from *in vitro* studies on both cultured mouse and human epithelial cells as well as from *in vivo* data. The enhanced lymphoproliferative capacity of thymocytes after coculture with epithelial cells is reduced when the latter are pretreated with TCDD. These studies also demonstrated that the human thymus may represent a target for the toxic action of TCDD with similar sensitivity to cells of a responsive mouse strain, showing the relevance of mouse data for human risk assessment. A second argument comes from mouse radiation chimeras with TCDD-responsive C57Bl/6 and TCDD-resistant DBA/2 strains, where TCDD-induced suppression of cytotoxic T-lymphocyte activity is determined by the host (epithelium) and not the donor (bone marrow, subsequently thymocytes). Third, TCDD has been shown to act on the thymic epithelium of rats, with formation of epithelial cell aggregates and the appearance of an unusual epithelial cell type and a more differentiated state of the cortical epithelium. Finally, as discussed in section 5.4.2.2.1, investigations of persons exposed to TCDD in Missouri suggest an association between TCDD exposure and the hormone secreting activity of thymic epithelium. Thus, TCDD-induced thymic atrophy may be explained by the inability of epithelial cells to provide the support needed to induce T-cell maturation and differentiation.

Apart from the effect on thymic epithelium, a direct action of TCDD on rat thymocytes has been shown in *in vitro* studies, resulting in a Ca-dependent endonuclease-mediated DNA fragmentation and cell death (apoptosis). However, from *in vivo* studies in which significant thymic atrophy was induced by TCDD, apoptosis appears not the most likely mechanism for *in vivo* thymocyte depletion. A direct action of TCDD on bone marrow stem cells has also been proposed and is substantiated by the finding that bone marrow cells of TCDD-treated donors manifest reduced capacity to populate the thymus of irradiated hosts. In addition to an effect on the thymus and hence on thymus-dependent immunity, TCDD also affects B lymphocytes, as manifested by suppressed antibody responses to thymus-independent antigens. This suppression occurring at higher concentrations, also appears to be AhR mediated.

From the studies discussed above, it can be concluded that TCDD and related compounds cause immune alterations, particularly of thymus-dependent immunity. Clear immuno-suppression with increased rate of infections was noted in the accidental poisoning in the Yu-Cheng and Yusho incidents. Following occupational exposure, effects are limited to one study in which a decrease was reported in T-helper cell function in adult workers as compared with a control group. Results of the Dutch PCB/dioxin study also suggest that background levels influence the human fetal and neonatal immune system, but the functional significance of the latter findings has to be established. The findings in man correlate in qualitative terms with the findings in experimental animals including the sensitivity of the developing immune system, illustrating the relevance of studies in laboratory animals. However, as exposure data on these mixtures of contaminants are mostly lacking for those individuals in which immune parameters were investigated, and because of the remarkable interspecies variation in toxicity of TCDD, quantitative assessment of the likelihood of effects of these chemicals on the human immune system remains difficult. Data that are of relevance in this respect are the above-mentioned studies using thymic epithelial cell cultures showing a similar sensitivity of cells of human and mouse origin. A study by De Heer et al. (1995) investigating the comparative sensitivity of the human thymus to TCDD indicated that the human thymus and the Wistar rat thymus display a similar sensitivity to TCDD, and also suggested that the human immune system is not as susceptible to the immunotoxic effects of TCDD as are very sensitive animal species such as the marmoset, a conclusion also reached by Golden et al. (1998). Regarding the adverse effects of TCDD in general in laboratory animals, immunotoxic effects, both adult and developmental, alongside developmental neurobehavioral (cognitive) effects, developmental reproductive effects (sperm counts, female urogenital malformations), and hormonal effects (endometriosis), were the most sensitive end points on a body burden basis (Van Leeuwen et al., 2000).

5.3.4 Conclusions and Recommendations on the Immune System

Of the large number of compounds with immunotoxic properties, only a few have been shown to cause immunotoxicity that is mediated through an endocrine-disrupting mechanism. These include the potent estrogen-receptor binding DES, which has been shown to cause a weak immunological change following *in utero* exposure, perhaps indicative of potential problems in immune regulation, and the AhR binding PCBs, PCDFs, and PCDDs. Immunotoxicity of DES occurred at pharmacological levels, raising the question of whether immunotoxic effects can be expected at all at low exposure levels to weak estrogens; absence of immune effects from methoxychlor supports this. PCBs, PCDFs, and PCDDs have been reported to alter immune parameters following accidental, occupational, and general population exposures. In particular, the latter findings need further study because they relate to possible effects on immune function from background exposure of the foetal and neonatal immune system. The reported data in humans for DES and for PCBs, PCDFs, and PCDDs are in line with studies in experimental animals. Because for the majority of immunotoxic chemicals, the mechanisms of action are unknown, further investigations are recommended including the study of endocrine-mediated immunotoxicity.

5.4 Cancer

5.4.1 Introduction

Increases in the incidence of certain cancers in many parts of the industrialized world are often cited as evidence that widespread exposure of the general population to EDCs has had adverse impacts on human health. Of particular concern are the observed increased incidences of cancers at hormonally sensitive sites, such as breast, uterus, prostate, and testis in Europe and North America. These increases cannot be adequately explained by improved diagnostic techniques, and it has been argued that these trends coincide roughly with the increasing use and release of industrial chemicals into the environment. Furthermore, these concerns are also based on plausible mechanisms of action because both human and experimental model studies have demonstrated that these cancers are either dependent on or modulated by the hormonal milieu. Under the multistage model of carcinogenesis (Russo and

Russo, 1996), chemicals are thought to act as tumor initiators, as tumor promoters, or as both. In this context, EDCs with estrogenic activity are generally regarded as tumor promoters.

This section focuses on the human data that bear on putative associations between EDCs found in the environment and risk of breast, uterus, prostate, testis, and thyroid cancer. The basic biology of these cancers and the etiologic role of hormones are very complex and beyond the scope of this review. Known risk factors for each cancer site are briefly discussed, emphasizing those factors that could indicate hormonal alterations. The section also integrates the human data with data from animal and experimental model carcinogenicity studies.

5.4.2 Breast Cancer

5.4.2.1 Human data.

5.4.2.1.1 Incidence and risk factors. The incidence of breast cancer increased steadily from the 1940s through the 1990s in many industrialized countries, with the highest risk found in Western Europe and North America. The increases may be due at least in part to increased screening. Relative frequencies vary fivefold among countries. Globally, the lowest rates occur in Asian countries and the highest in the USA and northern Europe (Kelsey and Berstein, 1996). In Japan and China, breast cancer rates are half as high as in the USA (Coleman et al., 1993). When women migrate from one country to another, their breast cancer rates become more similar to that of the host country over a period of two or three generations, suggesting the gradual influence of some environmental or lifestyle factor(s) (Stanford et al., 1995).

Extensive human studies support an etiological role for estrogens in breast cancer, all related to increased lifetime exposure to endogenous estrogen (Hulka and Stark, 1995). These include early age of menarche and/or late onset of menopause; not having children; postmenopausal obesity, which favors the conversion of androgens to estrogens in peripheral fat tissue (Siiteri, 1987); and alcohol consumption which boosts the amount of available E_2 (Reichman et al., 1993). Alternatively, breast cancer risk is markedly reduced by oophorectomy at a young age compared with natural menopause, a phenomenon not seen with other common cancers (Toniolo et al., 1995). The reduced breast cancer risk in Asian women living in Asia is accompanied by 40% reduction in lower serum estrogen levels compared to women in the USA and Great Britain (Key et al., 1990).

It has been suggested that altered reproductive patterns over the last 50 years, including fewer pregnancies and delaying pregnancy until later in life, have significantly increased the number of estrogen surges in modern women, resulting in increased breast cancer risk. In addition to endogenous sources of estrogen, pharmacological HRT and hormonal contraceptives are now commonly used. However, in the case of exogenous hormones, it should be recognized that the issue is not straightforward because for both HRT and oral contraceptives, the types of estrogen that have been used, the doses employed and whether and what type of opposing progestagen has been used are all relevant to the risk.

Hereditary factors also play an important role. A number of genes (e.g., BRCA1, BRCA2) have been associated with increased breast cancer risk (Miki et al., 1994; Lancaster et al., 1996; Li et al., 1997). Prevalence of at-risk genotypes varies widely, and ethnic and geographic differences may be of direct relevance to breast cancer risk. Population-based approaches are just beginning to address the effects of EDCs in high-risk populations carrying breast cancer susceptibility mutations (Ursin et al., 1997; Jernstrom et al., 1999; Brunet et al., 1998). The evidence supports a relationship between breast cancer and hormonal activity.

The U.S. National Toxicology Program has recommended that estrogen be added to the list of "potential human carcinogens." The IARC (1999) summarized studies on combined oral contraceptives and postmenopausal HRT as follows: "There is a small increase in the relative risk for breast cancer in current and recent users, but not for women by 10 years after they ceased using oral contraceptives." The cumulative data on long-term (>5 years) use of combined estrogen/progesterone HRT suggests that this combined therapy increases breast cancer risk (Schairer et al., 2000), but the data remain confusing. Physicians and patients remain faced with having to weigh the risk/benefits of treatment with HRT, contraceptives, or selective ER modulators (e.g., tamoxifen). Risks for endometrial and ovarian cancer are reduced by about 50% with combined oral contraceptive use, reductions persisting at least 10 years after cessation of use. The use of tamoxifen decreases the risk of breast cancer but increases the risk of uterine cancer (van Leeuwen et al., 1994).

5.4.2.1.2 Phytoestrogens. Dietary factors are known to contribute to breast cancer risk (Willett, 2001). One class of dietary compounds that has received much attention is phytoestrogens. Consumption of phytoestrogens, particularly soy products, is higher in Asia than in the Western World (Messina et al., 1994) and has been promoted as having potential anticancer protective effects or as an alternative to HRT. *In vitro* studies with human mammary epithelial (MCF-7) cells, for example, have shown that benzo[*a*]pyrene-induced proliferation is inhibited by genistein treatment and that genistein induces a significant increase in the apoptotic population of cells via a p53-mediated pathway. However, genistein at low concentrations can act as an inhibitor of proliferation in MCF-7 cells, which are estrogen dependent, and in MDA-468 human breast cancer cells, which are estrogen independent, whereas at higher concentrations it acts as an estrogen agonist in MCF-7 cells (Helferich et al., 1998). Other studies suggest that both proliferative and antiproliferative effects might be observed, depending on tumor cell type, dose, timing of phytoestrogen exposure, and phytoestrogen given (Aldercreutz and Mazur, 1997). This may be because phytoestrogens can act via multiple mechanisms of action, both ER mediated and non-receptor mediated, and compounds like genistein have been shown to possess both estrogenic and antiestrogenic properties (Chapter 3). This raises the question of whether foods rich in phytoestrogens may have complex actions in such diseases as breast cancer, exerting both preventive and promoting effects and perhaps depending on whether or not the tumor is estrogen dependent.

There is indirect evidence from both human and laboratory studies that high consumption of soy products may reduce the risk of developing breast cancer (Barnes, 1997). However, the evidence is inconsistent. Soy supplementation in women has been shown to increase the proliferation rate of breast cancer cells in premenopausal women (Petrakis et al., 1996; McMichael-Phillips et al., 1998). However, these studies contain potential confounding factors. Epidemiological studies on the relationship between soy consumption and risk of breast cancer are also inconsistent. A study in Singaporean women found that high soy intake was associated with a lower breast cancer risk among premenopausal but not postmenopausal women (Lee et al., 1991). Studies of Japanese and Australian women with higher concentrations of phytoestrogens in their blood and urine than women in Western countries showed a lower risk of breast cancer (Hirohata et al., 1985). However, a

similar study in women in Shanghai did not find significant differences in soy protein intake between breast cancer cases and their controls (Yuan et al., 1995). Four other studies also suggest that soy consumption is not associated with a reduced breast cancer risk (Hirohata et al., 1985; Messina et al., 1994; Yuan et al., 1995). A meta-analysis of currently available studies indicates that high soy intake might reduce the risk of developing premenopausal breast cancer but has no effect on postmenopausal breast cancer risk (Trock et al., 2000). Thus, caution is necessary in promoting the beneficial effects of phytoestrogens with respect to breast cancer (Bouker and Hilakivi-Clarke, 2000).

5.4.2.1.3 Organochlorine compounds. Although breast cancer is clearly an hormonally related disease, only 30–50% of all breast cancer cases can be attributed to increased life-long exposure to endogenous estrogens (Pike et al., 1993). The unknown etiology of the majority of breast cancer cases, along with large geographic differences in incidence rates, has shifted concerns to the potential role of environmental exposures, particularly exposures to EDCs. The majority of data relating environmental EDCs to human breast cancer are limited to persistent organochlorine compounds that have been identified throughout the world in human tissue, blood, and milk (Adami et al., 1995). Since the mid-1980s, a number of case–control studies have examined the potential association between exposure to organochlorines and breast cancer. These studies have been comprehensively reviewed elsewhere (Adami et al., 1995; Houghton and Ritter, 1995; NRC, 1999) and are therefore only briefly evaluated in this document. Studies published since 1990 are summarized in Table 5.11. The studies cited in Table 5.11 differ widely in terms of number of cases, exposure level, and time of exposure. For details on exposure levels, the reader is referred to the individual references.

DDT. The two main metabolites of DDT, *p,p*-DDE and *o,p*-DDT, have been identified in serum, adipose tissue, and breast milk of individuals with no history of occupational exposure and/or living in areas where DDT has not been used for years. This has raised concern about long-term exposure to DDT through food, which in turn could increase the risk for developing estrogen-dependent tumors such as cancer of the breast.

Around 34 published studies have considered the potential relationship between a nonoccupational exposure to DDT and female breast cancer (Table 5.11). Up to 1993, when Wolff and colleagues published their first study linking DDT exposure to breast cancer, the only available leads had come from four very small and incomplete case–control studies (Wasserman et al., 1976; Unger et al., 1984; Mussalo-Rauhamaa et al., 1990; Falck et al., 1992), and due to design flaws, there is no way to interpret their findings in a consistent manner. More recently, nine prospective case–control studies have failed to demonstrate that DDT, and more specifically *p,p*-DDE, increases the risk for breast cancer (Laden et al., 2001a, 2001b; Ward et al., 2000; Wolff et al., 2000a, 2000b; Hoyer et al., 1998, 2000; Dorgan et al., 1999; Helzlsouer et al., 1999; Hunter et al., 1997; Krieger et al., 1994). In addition, two retrospective case–control studies performed with postmenopausal women (among which breast cancer tends to be more estrogen dependent) did not find increases in breast cancer risk related to DDT exposure (vant'Veer et al., 1997; Moysich et al., 1998), nor did another 10 retrospective case–control studies including pre- and postmenopausal women (Millikan et al., 2000; Demers et al., 2000; Stellman et al., 2000; Aronson et al., 2000; Wolff et al., 2000a, 2000b; Zheng et al., 1999; Bagga et al.,2000; Mendonca et al., 1999; Dello Iacovo et al., 1999; López-Carrillo et al., 1997), in contrast to two positive studies (Romieu et al., 2000; Olaya-Contreras et al., 1998). A recent combined analysis of five U.S. studies showed no relationship between *p,p′*-DDE and breast cancer risk (Laden et al., 2001a, 2001b).

Methodological concerns that might confuse the relationship between DDT exposure and breast cancer were appropriately addressed by most of the recently published epidemiological studies. In general, breast-feeding history was taken into account during statistical analyses as a confounder, because lactation is a way to release the body's DDT burden and also has been shown to be a protective factor for breast cancer (Zheng et al., 1999). Adjustment for lipids in serum to improve serum DDE levels estimation was carried out in most of the recent studies, increasing the reproducibility of the findings and reducing the magnitude of random errors. A third feature has been the stratification by menopausal status or the restriction to postmenopausal breast cancer, to focus on the chance of ascertaining estrogen-dependent tumors, which are more prevalent after menopause.

Another source of concern is the potential existence of a threshold effect for DDE on breast cancer. If true, positive results could be expected from populations exposed to levels of DDT giving rise to serum DDE levels higher than 20,667 ng of DDE, which is the highest level of exposure reported in a nonoccupationally exposed population (Dorgan et al., 1999). However, no evidence of a link between DDT and breast cancer have been found in workers highly exposed to DDT (Cocco et al., 1997).

PCBs. Many of the studies on the relationship between PCB exposure and breast cancer have involved occupational exposure and have not found a positive association (Adami et al., 1995; Houghton and Ritter, 1995; NRC, 1999). In studies on general populations exposed to low levels of PCBs, none have detected a statistically significant increase in breast cancer risk, although at first sight the magnitude of some of the ORs might suggest an effect. The studies summarized in Table 5.11 are difficult to evaluate because PCBs are a mixture of congeners, containing both estrogenic and antiestrogenic compounds. Some studies focused on the concentration of a few individual congeners (e.g., PCB 118, PCB 138; Dorgan et al., 1999; Aronson et al., 2000); others sum the most important peaks of congeners according to their estrogenic potential (Sturgeon et al., 1998) or according to the percentage of chlorinated carbons (Moysich et al., 1998). Another approach has been to sum selected congeners or the total area under the curve and report them as the total amount of PCBs (Hoyer et al., 1998; Hunter et al., 1997; Krieger et al., 1994; Wolff et al., 1993; Moysich et al., 1998; Zheng et al., 1999). Overall, the data do not support an association between PCB exposure and increased breast cancer risk.

DIELDRIN, HCB, AND β-HEXACHLOROCYCLOHEXANE. there are limited data on the association between breast cancer and exposure to dieldrin, HCB, and β-hexachlorocyclohexane (Table 5.11). A study (Hoyer et al., 1998) including 268 cases from a cohort of 7,712 Danish women found a twofold increase in the risk of breast cancer associated with the highest plasma concentration of dieldrin. The relationship to dieldrin also showed a dose response. There were no associations with the other measured 45 compounds (28 of which were congeners of PCBs), and an endocrine mechanism has not been demonstrated.

In a prospective case–control study, after 9.5 years of follow-up, exposure to HCB induced a twofold increased risk of breast cancer among U.S. women diagnosed closer in time to blood study collection but not among women diagnosed later (Dorgan et al., 1999).

Table 5.11 - Summary of Studies on Selected EDCs and Breast Cancer

Reference *Biological Specimen*	Location	Design No. of Cases/ No. of Controls	OR (95% CI) or Mean Difference Cases–Controls (*p*-value)				
			p´p´-DDE	PCBs	β-HCH	HCB	Dieldrin
Laden et al., 2000b, 2001a *Serum*	11 states of USA	Nested case–control 381/381	High vs. low quintile 0.82 (0.49–1.37)	High vs. low quintile Total PCBs 0.84 (0.4–7.3)			
Wolff et al., 2000a, 2000b *Serum*	NYC, USA	Nested case–control 110/213	High vs. low quartile 1.30 (0.51–3.35)	High vs. low quartile Total PCBs 2.02 (0.76–5.37)			
Hoyer et al., 2000 *Serum*	Copenhagen, Denmark	Nested case–control 155/274	High vs. low quartile 1.4 (0.7–2.8)	High vs. low quartile 1.6 (0.8–3.3)	High vs. low tertile 1.2 (0.5–3.0)		
Ward et al., 2000 *Serum*	Norway	Nested case–control 150/150	High vs. low quartile 1.2 (NI)	High vs. low quartile Total PCBs 0.5 (NI)			
Millikan et al., 2000 *Serum*	North Carolina, USA	Case–control 292/270 (African-Americans)	High vs. low tertile 1.41 (0.87–2.29)	High vs. low tertile 1.74 (1.0–3.01)			
		456/389 (Whites)	High vs. low tertile 0.98 (0.67–1.43)	High vs. low tertile 1.03 (0.68–1.56)			
Demers et al., 2000 *Serum*	Quebec, Canada	Case–control 315/307 (population)	High vs. low quintile 1.00 (0.60–1.67)	High vs. low tertile PCB 153 1.28 (0.79–2.19)	High vs. low tertile 0.80 (0.47–1.35)		
		Case–control 315/219 (hospital)	High vs. low quintile 1.36 (0.71–2.63)	High vs. low quartile PCB 153 1.07 (0.54–2.12)	High vs. low tertile 0.83 (0.43–1.61)		
Wolff et al., 2000a, 2000b *Serum*	NYC, USA	Case–control 151/317	High vs. low tertile 0.93 (0.56–1.5)	High vs. low tertile High PCBs: 0.78 (0.45–1.13) Low PCBs: 0.96 (0.53–1.17)			
Stellman et al., 2000 *Adipose tissue*	New York State, USA	Case–control 232/323	High vs. low tertile 0.74 (0.44–1.25)	High vs. low tertile PCB (sum 14 congeners) 1.01 (0.60–1.69)			
Aronson et al., 2000 *Adipose tissue*	Ontario, Canada	Case–control 217/213	High vs. low quartile 1.62 (0.84–3.11)	High vs. low quartile Aroclor 1260 1.15 (0.58–2.25)	High vs. low quartile 0.69 (0.34–1.40)	High vs. low quartile 1.15 (0.57–2.34)	
Zheng et al., 2000 *Adipose tissue*	Connecticut, USA	Case–control 304/186	High vs. low quartile 0.9 (0.5–1.5)				
Romieu et al., 2000 *Serum*	Mexico City	Case–control 120/126	High vs. low quartile 3.81 (1.14–12.80) *p* for trend = 0.02				
Bagga et al., 2000 *Adispose tissue*	California, USA	Case–control 73/73	Per DDE unit 1.12 (0.79–1.6)				
Dorgan et al., 1999 *Serum*	Missouri, USA	Nested case–control 105/207	High vs. low quartile 0.8 (0.4–1.5)	PCB 118: high vs. low tertile ≤2.7 years of diagnosis: 1.4 (0.6–3.2) >2.7 years of diagnosis: 0.9 (0.4–2.4) PCB 138: high vs. low tertile ≤2.7 years of diagnosis: 1.9 (0.8–4.8). *p* for trend = 0.07 >2.7 years of diagnosis: 0.73 (0.3–1.6). *p* for trend = 0.07	High vs. low quartile 0.6 (0.3–1.3)	High vs. low tertile ≤2.7 years of diagnosis: 2.6 (1.1–6.2). *p* for trend = 0.02 >2.7 years of diagnosis: 0.6 (0.2–1.7)	High vs. low quartile 0.7 (0.3–1.3)
Helzlsouer et al., 1999 *Serum*	Washington, USA	Nested case–control 235/235 (1974) 105/105 (1989)	High vs. low quintile 0.10 (0.40–1.32) 0.58 (0.29–1.17)	High vs. low quartile Total PCBs (1974) 1.13 (0.59–2.15) High vs. low tertile Total PCBs (1989) 1.10 (0.38–1.51)			
Dello Iacova et al., 1999 *Serum*	Naples, Italy	Case–control 170/195	High vs. low tertile 1.24 (0.70–2.20)		Mean difference 0.27		
Mendonca et al., 1999 *Serum*	Rio de Janeiro, Brazil	Case–control 177/350	High vs. low quintile 0.83 (0.4–1.6)				
Hoyer et al., 1998 *Serum*	Denmark	Nested case–control 237/469	High vs. low quartile 0.88 (0.56–1.37)	High vs. low quartile Total PCBs 1.11 (0.70–1.77)			High vs. low tertile 2.05 (1.17–3.57) *p* for trend = 0.01

(Continued)

Table 5.11 - *Continued*

Reference *Biological Specimen*	Location	Design No. of Cases/ No. of Controls	OR (95% CI) or Mean Difference Cases–Controls (*p*-value)				
			p´p´-DDE	PCBs	β-HCH	HCB	Dieldrin
Moysich et al., 1998 *Serum*	Western New York, NY, USA	Case–control 154/192	High vs. low tertile 1.34 (0.71–2.55)	High vs. low tertile Total PCBs 1.13 (0.61–2.15) Selected PCBs 1.34 (0.72–2.47)		High vs. low tertile 0.81 (0.43–1.53)	
Olaya-Contreras et al., 1998 *Serum*	Bogota, Colombia	Case–control 153/153	High vs. low quartile 1.95 (1.10–3.52)				
Hunter et al., 1997 *Serum*	Boston, USA	Nested case–control 236/236	High vs. low quintile 0.72 (0.37–1.40)	High vs. low quintile Total PCBs 0.66 (0.32–1.37)			
van't Veer et al., 1997 *Adipose tissue*	Germany, The Netherlands, N. Ireland, Swizerland, Spain	Case–control 265/341	High vs. low quartile 0.48 (0.25–0.95) *p* for trend = 0.02				
Lopez-Carrillo et al., 1997 *Serum*	Mexico City	Case–control 139/139	High vs. low tertile 0.76 (0.41–1.42)				
Krieger et al., 1994 *Serum*	California, USA	Nested case–control 150/150	High vs. low tertile 1.33 (0.68–2.62)	High vs. low tertile Total PCBs 0.94 (0.48–1.84)			
Wolff et al., 1993 *Serum*	New York, NY USA	Nested case–control 58/171	High vs. low quintile 3.68 (1.01–13.5) *p* for trend = 0.04	High vs. low quintile Total PCBs 4.35 (0.9–20.0)			
Stellman et al., 2000 *Adipose tissue*	New York, NY USA	Incomplete case–control 5/5	Mean difference 219	Mean difference 100	Mean difference 2.4	Mean difference 6.1	
Liljegren et al., 1998 *Adipose tissue*	Sweden	Incomplete case–control 43/35	High vs. low tertile 0.4 (0.1–1.2)	High vs. low tertile Total PCBs 0.7 (0.1–2.4)		High vs. low quintile 1.3 (0.3–4.5)	
Guttes et al., 1998 *Adipose tissue*	Germany	Case–control 45/20	Mean difference 62 ($p = 0.01$)	Mean difference PCB 118 = 25 ($p = 0.04$) PCB 153 = 24 ($p = 0.08$)	Mean difference –18 ($p = 0.36$)	Mean difference 18 ($p = 0.40$)	
Schechter et al., 1997 *Serum*	Vietnam	Incomplete case–control 21/21	High vs. low tertile 1.14 (0.23–5.68)				
Sutherland et al., 1996 *Serum*	South Carolina, USA	Incomplete case–control 20/17	Mean difference ER+ 1,366.9 ($p = 0.01$) ER– 156.4 ($p = 0.63$)	Mean difference PCB 99 = 10.2 ($p = 0.05$)			
Dewailly et al., 1994 *Serum*	Quebec, Canada	Incomplete case–control 20/17	Mean difference ER+ 1,366.9 ($p = 0.01$) ER– –156.4 ($p = 0.63$)	Mean difference Total PCBs ER+ 7.7 ($p = 0.79$) ER– –65.5 ($p = 0.39$)	Mean difference ER+ 0 ($p = 0.77$) ER– 5 ($p = 0.92$)	Mean difference ER+ 8.3 ($p = 0.29$) ER– –2.3 ($p = 0.53$)	
Falck et al., 1992 *Adipose tissue*	Connecticut, USA	Incomplete case–control 20/20	Mean difference 703 ($p = 0.04$)	Mean difference Total PCBs 570 ($p = 0.02$)			
Mussalo-Rauhamaa et al., 1990 *Adipose tissue*	Finland	Incomplete case–control 44/33	Mean difference 0.02 ($p = 0.87$)	Mean difference Total PCBs 0.25 ($p = 0.17$)			
Unger et al., 1984 *Adipose tissue*	Denmark	Incomplete case–control 18/35 (autopsies) 14/21 (biopsies)	Mean difference NI (autopsies) 0.02 (biopsies)	Mean difference 1.35 (autopsies) –0.04 (biopsies)			
Wasserman et al., 1976 *Adipose tissue*	São Paulo, Brazil	Incomplete case–control 9/5	Mean difference –3.95	Mean difference∫ 6.15 ($p < 0.01$)	Mean difference 0.22		Mean difference 0.616

NI, no information available.

TCDD AND PBBS. Accidental and/or occupational exposures to the industrial chemical contaminant TCDD and industrial PBB compounds have also been investigated with respect to increased risk of breast cancer. Two studies of women exposed to PBBs in Michigan showed equivocal results (Henderson et al., 1995; Sinks et al., 1996). Women exposed to TCDD in Seveso, Italy, had a decreased risk of breast cancer (Bertazzi et al., 1993), but the number of cases was very small. Two occupational studies on TCDD exposure have shown equivocal results (Manz et al., 1991; Kogevinas et al., 1994).

5.4.2.1.4 Timing of exposure. As noted in Chapter 2, the time of life when exposures take place may be critical to defining the dose–response relationships of EDCs for breast cancer as well as for other health effects. The development of the mammary gland occurs in multiple stages. Fetal development of the mammary gland rudiment is governed by tissue interactions in both males and females. In females, the pubertal period drives ductal morphogenesis, and pregnancy results in massive differentiation of the mammary gland. Thus, the perinatal period and the period between age at menarche and age at first full-term pregnancy may be particularly important for breast tumor development and latency (Snedeker and Di Augustine, 1996). Young girls exposed to carcinogenic agents during puberty may be at high risk of future breast cancer due to susceptibility of rapidly growing breast tissue mediated by hormonal changes during this time. This claim is supported by data from atomic bomb survivors, where an increased risk of breast cancer was found in women exposed before 20 years of age (Tokunaga et al., 1987). Similarly, an elevated risk was found for women irradiated during childhood for medical reasons (Hildreth et al., 1989). Cigarette smoking during prepubertal years may also be related to an increased risk of developing future breast cancers (Palmer et al., 1991). Increased risk was attributed to the age when the girls began smoking rather than the duration of smoking.

The perinatal period may also be a susceptible period for exposures and future breast cancer risk. Some studies have shown that women taking DES during pregnancy to prevent miscarriage have been shown to have a slightly increased risk of developing breast cancer 30 years after taking the drug (Colton and Greenberg, 1993). A recent combined analysis (Titus-Ernstoff et al., 2001) of two cohorts of women demonstrated a modest association between DES exposure and breast cancer risk (risk ratio = 1.27). There was no evidence that DES was associated with risk of ovarian endometrial or other cancers. Data on the risks of breast cancer in daughters of DES-exposed women are not yet available.

Studies of Japanese women with latency periods as long as 35 years found a ninefold greater risk of breast cancer in the subgroup of women who were younger than 4 years of age during the atomic bombing (Tokunaga et al., 1987). Studies in migrants also suggest that the influence of dietary factors in breast cancer is greatest during early childhood and adolescence (Lipworth, 1995).

5.4.2.2 Animal and experimental data. Experimental animal models have provided useful tools for answering specific questions on the biology of mammary cancer relative to their validity on the human disease, as well as for exposure to toxic chemicals (Russo and Russo, 1996). However, there are significant differences in the development of mammary gland neoplasia among species and among strains within a species, and experimental data must be interpreted with caution.

5.4.2.2.1 Synthetic hormones, phytoestrogens, and other estrogens. Experimental studies have clearly shown that hormone treatment can alter mammary gland morphology and tumor sensitivity in rodents and that the observed effects are dose dependent. Studies in female beagle dogs have shown that progestin exposure induces hyperplastic changes in the mammary ductal epithelium (van Garderen et al., 1997) and may increase breast cancer risk (Skegg, 1995). Medroxyprogesterone acetate when administered to rodents, cats, and monkeys, alone or in combination with estrogens, results in an increased risk of mammary cancer development (Rutteman, 1992; Skegg, 1995). Tamoxifen has been shown to protect against mammary gland tumors in various strains of rats (Jordan, 1991; Kotoula et al., 1993; Ménard et al., 2000).

As noted above, phytoestrogens can have a variety of endocrine-modulating effects and properties (Barnes, 1998a, 1998b). Experimental studies support the human observations that timing of exposure may be the critical factor in determining the effects of phytoestrogens. For example, isoflavones found in soy products have been generally thought to confer reduced breast cancer risk. In seven out of nine animal studies, a lower number of tumors were observed in rats whose diet was supplemented by soy (Barnes, 1997). However, genistein administration in rats during gestation results in a dose-dependent increase in mammary tumor susceptibility in the F_1 animal (Hilakivi-Clarke et al., 1999a, 1999b). Immature rats given subcutaneous injection or infusion of the estrogenic compound bisphenol A show mammary gland proliferation (Colerangle and Roy, 1997).

Experimental models also offer tools to assess the interaction between genetic factors and environmental exposures on breast cancer risk. *In vivo* studies, using mice heterozygous for the breast cancer susceptibility genes, BRCA1 and BRCA2, showed that exposure to DES for 26 weeks (beginning a week before ductal morphogenesis) led to inhibited growth and differentiation of ducts, suggesting compromised DNA repair processes (Bennett et al., 2000).

5.4.2.2.2 Organochlorine compounds and genotoxic agents. The evaluation of chemicals in 2-year bioassays in laboratory rodents has become the cornerstone for identifying those chemicals most likely to cause cancer in humans (Huff et al., 1991a, 1991b). However, these carcinogenesis assays do not include exposure during pregnancy and lactation that can influence expression of mammary gland carcinogenesis (Grubbs et al., 1985). The mammary gland is a frequent source of tumors or neoplasms in laboratory rodents. They may occur spontaneously and are also induced by genotoxic agents. The long latency and variable incidence of mammary tumors in rodent strains limit the utility of studying such tumors as models of human disease (Neumann et al., 1996). Genotoxic agents such as dimethylbenz(*a*)anthracene and 3-methylcholanthrene are commonly used to elicit mammary gland tumors in rodents. These carcinogenic agents are thought to act as initiators within the context of a multistage model of carcinogenesis (Russo and Russo, 1996). Following initiation with such genotoxic agents, hormonal factors may promote tumor formation. Many of these chemicals have not been strongly linked to breast cancer in epidemiological studies.

Although there has been suspicion about involvement of organochlorine compounds in human breast cancer development, these compounds have not generally induced mammary cancer in animals. Chemicals measured include pesticides—DDT and its major metabolite DDE, chlordane, HCB, benzene hexachloride (or lindane), and halogenated biphenyls—as well as PCBs, PBBs, and TCDD. With the exception of DDT, none of these compounds has been associated with mammary gland cancer in animals, although other site-specific cancers have been identified (Wolff et al., 1996).

The triazine herbicide, atrazine, has been reported to promote mammary tumor growth in a certain strain of rats, but the mechanism of this response is not relevant to breast cancer in humans (Chapter 3, section 3.13).

5.4.2.3 Conclusions and recommendations on breast cancer. Although numerous human epidemiological studies have been conducted to determine whether environmental EDCs may contribute to an increased risk of breast cancer, the results remain inconclusive. Overall, the current scientific evidence (from human and animal studies) do not support a direct association between exposure to environmental EDCs and increased risk of breast cancer. However, all the studies published to date have measured EDC exposure levels in adult women. The claim that the time of life when exposure takes place (e.g., prenatal, neonatal, childhood, adolescence) may be the most critical factor is supported by human data on radiation and smoking and by basic research in animal models. Adult women currently at risk for breast cancer may have been exposed to exogenous EDCs *in utero* or during infancy, childhood, and adolescence in the mid-1900s when contaminant levels of organochlorines were higher. Research is urgently needed to address the role of timing of exposure. Because human prospective studies would be complex, time-consuming, and expensive, researchers should be encouraged to utilize and develop animal models to address this important issue and to use serum banks from this time and conduct retrospective follow-up studies.

Breast cancer is likely due to many factors, including genetics, lifestyle, diet, endogenous hormone status, and environmental factors. Research on whether potential complex interactions among these factors, modulated by individual genetic susceptibility factors, produce breast cancer is critical. Until consistent and compelling data on these issues become available, the role of EDCs in contributing to breast cancer incidence is likely to remain a highly controversial issue.

5.4.3 Endometrial Cancer

5.4.3.1 Human data. The uterus is highly responsive to hormonal alterations. Cancer of the uterus is more common in developed countries, with a similar pattern of hormonal risk factors as breast cancer. There is clear evidence that unopposed estrogen is the major risk factor for endometrial cancer (IARC, 1999; Potischman et al., 1996). However, there is no increasing temporal trend for endometrial cancer.

Epidemiologic data on the effects of environmental EDCs on endometrial cancer are limited. Sturgeon et al. (1998) found no association between endometrial cancer and 27 PCB congeners, 4 DDT-related compounds, and 13 other organochlorine compounds. Several retrospective occupational cohort studies also observed no association (Bertazzi et al., 1987; Brown, 1987; Sinks et al., 1996). In the Seveso industrial accident, TCDD exposure appeared to reduce the risk of uterine cancer, but the number of cases was small (Bertazzi et al., 1993).

There is some evidence that dietary isoflavones protect from endometrial proliferation, as shown by a decreased incidence of endometrial cancer in Japanese and U.S. (Hawaiian) women consuming isoflavone-rich diets. Specifically, high consumption of soy products and other legumes was associated with a decreased risk of endometrial cancer for the highest compared with the lowest quartile of soy intake (p for trend = 0.01; OR, 0.46; 95% CI, 0.26–0.83).

5.4.3.2 Experimental and animal data. Numerous studies have characterized the estrogenic potential of environmental chemicals using *in vitro* endometrial cancer cell model systems or *in vivo* classical uterotropic assay (Klotz et al., 1997; Hunter et al., 1999). Certain phytoestrogens, such as genistein and daidzein (Santell et al., 1997; Boetger-Tong, 1998), and some environmental chemicals (e.g., methoxychlor, nonylphenol, bisphenol A) have all been shown to induce a uterotropic response in rodents (Odum et al., 1997; Ashby, 1998). Neonatal treatment of mice on PNDs 1–5 with either DES or the phytoestrogen, genistein, has been shown to cause uterine adenocarcinoma by 18 months (Newbold et al., 2001). Though others have shown that soy isoflavones inhibit E_2-mediated endometrial proliferation in macaque monkeys (Cline and Foth, 1998), which is consistent with the human study mentioned above. This is perhaps not surprising in the case of phytoestrogens that have both estrogenic and antiestrogenic effects (Whitten and Patisaul, 2001).

However, as discussed in Chapter 3, there are few data on whether long-term exposure to these chemicals at low doses can result in neoplasms. Long-term exposure in rats to toxaphene (Reuber, 1979) and methoxychlor (Reuber, 1980) resulted in uterine hyperplasias. Exposure to the antiandrogenic herbicides (atrazine and vinclozolin) at high doses increased uterine adenocarcinoma development in rats (IARC, 1991; Mellert, 1995).

As with other cancers, the timing of exposure is critical to the potential development of uterine cancer. Developmental exposure of mice to DES results in uterine neoplasms, but treatment of adult mice with comparable levels of DES does not induce uterine neoplasms (Newbold et al., 1991).

5.4.3.3 Conclusions and recommendations on endometrial cancer. Because endometrial tissue is very responsive to the actions of antiestrogenic and estrogenic compounds, it should be a sensitive target tissue for EDC action. However, neither limited human data nor animal studies currently support an association between exposure to organochlorines and risk of endometrial cancer.

5.4.4 Testicular Cancer

5.4.4.1 Human data. Testicular cancer is the most common malignancy among young men, 25–34 years old (Adami et al., 1994). Most of the tumors occurring in young men are seminomas of germ cell origin; hence, early exposures may be relevant. Toppari et al. (1995) have estimated that testicular cancer incidence in men under 50 has increased 2–4% per annum since the 1960s in many developed countries, and in countries with a long history of cancer registration, the start of the increase can be traced back to around 1920 (Bergstrom et al., 1996). There are marked differences in incidence levels of testicular cancer between countries and between races. The incidence in Denmark is about fourfold higher than in neighboring Finland, which parallels observations of higher sperm counts, a low incidence rate for hypospadias, and lack of temporal increase in hypospadias in Finland (sections 5.1.3.1 and 5.1.6.4). In the USA, Caucasians have about a threefold higher incidence than do African Americans. Cryptorchidism (section 5.1.7) is a known risk factor for testicular cancer, suggesting a possible prenatal etiology (Moss et al., 1986) or, as some have suggested, there may be modulation by early postnatal exposure to estrogens or antiandrogens (Bergstrom et al., 1996; Ekbom et al., 1996; Moller and Skakkebaek, 1999).

No single study of sons of DES mothers has shown a significant increase in testicular cancer, but a meta-analysis of available studies concluded there was an overall increase of approximately twofold, which was just statistically significant (Toppari et al., 1996).

There are currently no published epidemiological studies of testicular cancer in which blood concentrations of environmental

EDCs have been measured. A recent regression analysis (Cocco and Benichou, 1998) used *p,p*-DDE concentrations from human adipose tissues obtained in 1968 to predict testicular cancer among white males in 22 U.S. states, found no association between the antiandrogen DDE and testicular cancer some 2–22 years later.

5.4.4.2 Experimental data. The type of testicular cancer commonly found in humans, seminomas, which are preceded by atypical intratubular germ cells termed CIS, is extremely rare in laboratory animals. The testicular tumors in rodents induced by some chemicals are Leydig cell tumors (Cook et al., 1999). Thus, until recently, experimental animal models appropriate for extrapolation to humans were lacking. However, atypical germ cells resembling human CIS cells have now been reported in cryptorchid stallions (Veeramachaneni and Sawyer, 1998; Veeramachaneni, 2000) and in an infertile rabbit (Veeramachaneni and VandeWoude, 1999), in association with developing tubular seminomas. In further studies, similar CIS lesions have been induced in rabbits exposed in utero and/or in infancy to the EDCs octylphenol, *p,p′*–DDT/DDE or zeranol (Veeramachaneni, 2000). These treatments also resulted in a variable incidence of undescended testes with CIS-like cells in both undescended testes and scrotal testes, but surgically induced cryptorchidism did not result in CIS, showing that the germ cell atypia was induced by the chemical exposure and not by abdominal retention of the testes. *In utero* exposure to dibutyl phthalate in the rabbit has also been shown to result in undescended testes, ambiguous genitalia, hypospadias, regressed prostate, and missing bulbourethral glands (Higuchi et al., 1999), analogous to effects seen following treatment with phthalates in the rat (Chapter 3). These observations are relevant because cryptorchidism is a known risk factor for CIS in humans, and they illustrate the potential relevance of the rabbit as a model for this type of cancer in humans.

5.4.4.3 Conclusions and recommendations on testicular cancer. Risk factors for testicular cancer are associated with disorders of androgen production or action. There are also limited data from animal studies that exposure of the male fetus to high levels of estrogen may increase the risk of developing testicular cancer. However, there are no published analytical epidemiologic studies that examine a connection between exposure to estrogenic and/or antiandrogenic (e.g., DDE) compounds and testicular cancer. Moreover, validated animal models for germ cell (testicular) tumors observed in man currently do not exist. Increased research efforts are needed to develop suitable animal models for testicular cancer, so that the effects of prenatal and postnatal exposure to EDCs can be investigated. Although the data are limited, some evidence suggests that the incidence of cryptorchidism and hypospadias may show similar geographic differences to the incidence of testicular cancer. The potential roles of other environmental factors (e.g., diet, occupational exposures) in producing testicular cancer are unknown and need to be investigated.

Efforts to obtain data regarding the possible existence of a testicular dysgenesis syndrome, of which reduced sperm quality, cryptorchidism, and testicular cancer are components, are also needed, along with research on possible shared etiologies.

5.4.5 Prostate Cancer

5.4.5.1 Human data. Prostate cancer is the most commonly diagnosed cancer among men in developed countries (Parker et al., 1997). Since the mid-1980s, age-adjusted incidence rates have increased abruptly, which can be largely attributed to improved screening and diagnostic tests. However, what accounts for the long-term rise in both incidence and mortality is not known. There are racial differences in susceptibility, with the incidence being rare in Asians, 20–30 times higher in Caucasians, and even higher in African-American males (Crisp et al., 1998).

Little is known about the causes of prostate cancer, but it is both hormone dependent and able to be modulated by hormone treatment (section 5.1.6.5). A few small epidemiological studies on men in Japan and Japanese migrants to the USA indicated a tendency for increased soy consumption to be associated with lower prostate cancer risk (Morton et al., 1997). The limited epidemiologic data on potential associations between prostate cancer and exposure to environmental EDCs are derived mainly from occupational exposures, and all of them lack internal exposure information. Occupational studies of PCB-exposed workers have not shown an association between PCBs and prostate cancer (Bertazzi et al., 1987; Brown, 1987; Sinks et al., 1992). No significant increases of prostate cancer were reported as a result of accidental TCDD exposure in Seveso, Italy (Bertazzi et al., 1993). Similarly, in the cohort study based on the international registry of workers exposed to TCDD, there was no increased prostate cancer mortality (Saracci et al., 1991). Other studies on workers in Germany (Becher et al., 1996) and the USA (Fingerhut et al., 1991), showed a small but statistically insignificant excess in prostate cancer mortality, based on a limited number of cases. The regression analysis (Cocco and Benichou, 1998) using DDE concentrations of adipose tissues (obtained in 1998) also indicated no positive association between DDE and prostate cancer mortality. In a retrospective cohort epidemiology study of Canadian farmers linked to the Canadian National Mortality Database, a weak but statistically significant association between acres sprayed with herbicides and prostate cancer deaths was found (Morrison et al., 1993). However, the possible role of chemical exposure and endocrine disruption as a contributing factor in the etiology of adenocarcinoma of the prostate cannot be excluded.

5.4.5.2 Experimental data. Experimental research on the etiology of prostate cancer has been hindered by the lack of suitable animal models for study (Bosland, 1992). Rodent models are of uncertain relevance to man. In contrast to its frequent occurrence in humans, prostate cancer is rare in laboratory rodents, but the frequency can increase significantly with hormone treatment depending on the strain of rat, dose of hormone, and duration of treatment (Bosland, 1992). Dogs can develop prostate cancer, but further research is needed to determine its relevance to humans. There are few experimental studies on the effects of phytoestrogens on prostate cancer. In three animal studies, the effects of soy showed reduced tumorigenesis (Lee et al., 1991; Messina et al., 1994). Genistein has been shown to inhibit chemically induced prostate cancer in rats (Pollard et al., 2000). The green tea polyphenol EGCG is known to rapidly reduce the size of human prostate and breast tumors grown in nude mice, and its been hypothesized that consumption of green tea may contribute to the lower mortality from these tumors in some Asian countries. A recent study showed that EGCG may inhibit androgen action by repressing transcription of the AR gene (Ren et al., 2000). Very few chemicals have been identified that can induce prostate cancer in the 2-year bioassay studies (Huff et al., 1991a, 1991b). Transgenic mouse models for prostate cancer have been developed and offer opportunities to study hormone-responsive elements and the effects of chemicals on the multistage progression of prostate cancer (Gingrich et al., 1996). Future studies using these models may provide additional information on the etiology of prostate cancer.

5.4.5.3 Conclusions and recommendations on prostate cancer. It is known from experimental data (section 5.1.6.5.1) that the development of the prostate gland or the propensity of this organ to develop cancer can be affected by perinatal/postnatal exposure to estrogens and phytoestrogens and possibly also androgens and AhRs. The few epidemiologic studies on prostate cancer do not include measurements of dose in body fluids or tissues. Studies on PCB, TCDD, and DDT exposures showed no association with increased prostate cancer. Exposure to herbicides or polyaromatic hydrocarbons has been linked to prostate cancer, but the evidence is weak, the mechanism is unknown, and more research is needed. Little is known about the effects of other environmental factors (e.g., genetics, diet, endocrine status) on the incidence of prostate cancer.

5.4.6 Thyroid Cancer

5.4.6.1 Human data. As mentioned in Chapter 3 and section 5.3, the thyroid gland plays a key role in numerous endocrine and metabolic and physiological functions. The thyroid hormones are particularly important to processes involving growth and development and some environmental chemicals (e.g., certain PCBs) have been shown to possess antithyroidal activity (Porterfield and Hendry, 1998). Thyroid hormones are also involved in the carcinogenic process and can affect tumor formation, growth, and metastasis (Guernsey and Fisher, 1990). Thyroid cancer is an uncommon and largely nonfatal tumor with incidence rates two to three times higher in females than in males (Landi et al., 1998). Nordic countries appear to have the highest incidence rates (Coleman et al., 1993). In contrast to clinically apparent disease, small occult thyroid tumors are noted at autopsy in up to about 50% of cases surveyed. The only known human thyroid carcinogens are x-rays and ionizing radiation (NRC, 1990; Lomat et al., 1997). Persons living in iodide-deficient areas of the world are unable to synthesize adequate levels of thyroid hormones and develop hyperplastic thyroid lesions. There is conflicting evidence whether thyroid cancer is increased in these individuals (Galanti et al., 1995). In epidemiologic studies, goiter and thyroid nodules have been shown to be risk factors for thyroid cancer (Ron et al., 1987). Graves' disease and Hashimoto's disease often precede thyroid carcinoma and may be part of the causal pathway. There is some evidence that sustained stimulation of TSH receptors is important for development of thyroid cancer in chronic goiter cases (Shi et al., 1991). To date, no environmental chemical has been identified as being carcinogenic to the human thyroid. The etiology of thyroid cancer in humans is largely unknown, and limited trend data are available.

5.4.6.2 Experimental data. Rodents and humans share a common physiology in regard to the hypothalamic-pituitary-thyroid feedback system, and the thyroid is a commonly affected target organ in rodent chemical carcinogenicity studies (Huff et al., 1991a, 1991b). In a review of potential carcinogenicity of 240 pesticides, at least 24 (10%) produced thyroid follicular cell tumors in rodents (Hurley et al., 1998). Mutagenicity does not appear to be a major determinant in thyroid carcinogenicity for pesticides (except for possibly acetochlor), in contrast to some other chemicals, such as aromatic amines (Hill et al., 1989). The mechanism of action by nongenotoxic agents is thought to be due to a sustained increase in serum TSH levels (Kanno et al., 1996), which can occur through various perturbations of the hypothalamic-pituitary-thyroid axis (section 3.5). The most potent thyroid carcinogens are TPO inhibitors, which can cause a drastic decrease in serum thyroid hormone levels and trigger TSH hypersecretion from the pituitary gland via release from negative feedback. Highly potent TPOs are thionamides (thiourea, ethylene thiourea, propylthiouracil, etc.) and aminotriazole (Hill et al., 1989). It is also postulated that during these reactions, free radicals are generated that interfere with other enzymes and bind to other proteins and possibly to DNA (Krauss and Eling, 1987). Other chemicals (e.g., the pesticides clofenzetine, fenbuconazole, pentachoronitrobenzene) appear to enhance the hepatic metabolism and excretion of thyroid hormones.

The environmental chemicals TCDD, PCBs, and PBBs also increase the metabolism of thyroid hormones, resulting in potential increases in thyroid neoplasms (Barter and Klaassen, 1992). PCBs have also been shown to block the binding sites for T_4 to serum transport proteins that causes enhanced clearance from serum and decreased availability to tissues (Brouwer and Van den Berg, 1986). PCBs do not bind directly to the thyroid receptor (Cheek et al., 1999).

5.4.6.3 Conclusions and recommendations on thyroid cancer. A direct association between exposure to specific EDCs and thyroid cancer is not supported by human experimental data. However, some EDC chemicals can affect the hypothalamic-pituitary-thyroid axis, and the basic mechanisms of interaction among various hormonal systems need to be elucidated to understand the process of thyroid carcinogenesis in humans.

5.4.7 Conclusions and Recommendations on Cancer

Although there is biological plausibility and some experimental evidence that EDCs may contribute to hormonally influenced human cancer, the current state of the science has not provided clear evidence for a causal link. In the case of testis cancer, human studies have not yet explored this possible link. Where possible associations with EDC exposure have been explored (mainly for breast cancer), the overall strength of the evidence of a causal association is weak. However, there is not enough information to completely reject the hypothesis that endocrine disruptors such as PCBs, dieldrin, or some other not yet evaluated compound(s) could play a role in the incidence of (female and/or male) breast, endometrial, prostatic, and testicular malignant tumors. Further research should focus on the assessment of exposure to endocrine disruptors during critical periods of human development (intrauterine life, adolescence, etc.), in relation to the occurrence of cancer at endocrine-sensitive sites during childhood or at later stages of life. Cancer registries will continue to provide useful information on geographical and temporal trends in cancer incidence that could be exploited for hypothesis testing.

5.5 Other Endocrine Systems Potentially Vulnerable to EDCs

In this chapter, the main focus has been on the reproduction, central nervous and immune systems, and cancer at endocrine sites. It is clear, however, that endocrine disruption in its broadest sense encompasses more than just these targets and may involve hormones other than sex and thyroid hormones. The potential effects of EDCs on other hormones and their target organs, such as growth hormone, insulin, and adrenocortical hormones, are not reviewed in detail here because the available research to date is very limited. The physiological roles of some of these other hormones are outlined in Chapter 3, indicating the scope for adverse effects should these systems prove vulnerable to EDCs. As an illustration of exploratory work underway on other endocrine systems, a brief review of glucocorticoids is given below.

The developmental and functional endocrinology of the hypothalamic-pituitary-adrenal axis is described in Chapter 3 (section 3.4). Glucocorticoids bind to intracellular GRs, which belong to the same receptor family as the sex hormone receptors. The similarities between these two systems are many, for example, with respect to regulation of synthesis and the functioning of the receptors, indicating that the glucocorticoid system also may be vulnerable to disturbance by xenobiotics. Most cells contain GR, but they are especially abundant in target organs such as the liver, the hypothalamus, the pituitary, and the hippocampus. Given the many functions of glucocorticoids in the body (metabolic, cardiovascular, developmental, immunosuppressive, anti-inflammatory), the potential for interference by EDCs is evident. In normal fetal development, for example, glucocorticoids are responsible *inter alia* for pulmonary surfactant secretion and the regulation/differentiation of neural crest cells of the nervous system. Excessive and prolonged exposure to glucocorticoids, such as during chronic stress, has recently been shown to induce neurodegeneration in the hippocampus, a brain structure involved in learning and memory processes; similarly, effects on behavior are observed in GR knockout mice (De Kloet at al., 1998, Sapolsky, 1996).

Experimental studies have indicated several mechanisms whereby xenobiotics can interfere with glucocorticoid homeostasis. The best known is the cytotoxicity exerted by the DDT metabolite DDD in adrenocortical cells (Nelson and Woodard, 1949; Adamson et al., 1973); *o,p′*-DDD has therefore been used in the past as a drug to suppress cortisol secretion. Methylsulfonyl-DDE is another DDT metabolite that is adrenotoxic (Lund et al., 1988). In mice, a single administration of 12 mg/kg body weight results in a decreased capacity to synthesize corticosterone that remains for at least 40 days (Jönsson, 1994). The mechanism involves bioactivation by CYP11b1 (a mitochondrial enzyme only present in the adrenal cortex), mitochondrial damage, and finally cytotoxicity (Lund and Lund, 1995; Jönsson et al., 1991).

Inhibition of adrenocortical enzymes may also result in disturbed glucocorticoid homeostasis. Azole compounds, discussed above in relation to inhibition of cytochromes involved in synthesis of sex hormones (e.g., aromatase inhibitors; see Chapter 3, sections 3.12.5.2 and 3.12.5.3), are also likely to affect the glucocorticoid pathway. Several pharmaceuticals (e.g., ketoconazole and metyrapone) have been shown to be potent inhibitors of glucocorticoid synthesis (Couch et al., 1987). There is also scope for interactions between the glucocorticoid and sex hormone synthesis pathways because the first steps are identical, involving transformation of cholesterol via (17-hydroxy-)pregnenolone into (17-hydroxy-)progesterone. There are data indicating that inhibition of adrenocortical enzymes mediating the further metabolism of (17-hydroxy-)progesterone may lead to an increased formation of sex hormones. Mechanistic *in vitro* studies have shown that a shift from glucocorticoid to androgen production is caused by the increased availability of substrate for the production of sex hormones (Mesiano et al., 1999). Thus, the adrenocortical production of androstenedione and dehydroepiandrosterone is normally rather insignificant but may become important in pathological conditions and at different life stages, such as at adrenarche (Papadimas, 1997).

Finally, xenobiotics may also interfere with glucocorticoid homeostasis by interacting with the GR. Studies have shown that xenobiotics may both up- and down-regulate the density of GR in organs (Budziszewska et al., 1995; Bellingham, 1992). Recent *in vitro* data also suggest that xenobiotics may bind directly to the GR. Johansson et al. (1998) studied 24 ubiquitous methylsylfonyl-PCBs and found that, at micromolar concentrations, some methylsylfonyl-PCBs compete with dexamethasone in binding to the GR. One of them was studied in Chinese hamster ovary cells carrying the human GR and a reporter gene, which indicated an antagonistic effect of 3-methylsulfonyl-2,5,6,2′,4′,5′-hexachlorobiphenyl at the GR.

These observations in experimental models are supported by wildlife observations, which indicate that perturbations of the glucocorticoid system do occur. Canadian studies have consistently shown that fish from polluted waters have a decreased capacity to synthesize cortisol in response to stress (Hontela, 1998). Lorentzon et al. (1999) have recently shown that current levels of organochlorines in herring gull (*Larus argentatus*) embryos are inversely related to blood corticosterone concentration and activities of corticosterone-dependent intermediary metabolic enzymes. The finding that DDT and corticosterone induce similar pathological development of the upper mandible in tadpoles has raised the question of whether DDT mimics corticosterone or acts via corticosterone by inducing stress that subsequently increases the levels of corticosterone (Hayes et al., 1997).

Given the above findings in laboratory animals and in wildlife, it is clear that there is potential for effects on glucocorticoid homeostasis from certain EDCs such as DDT and PCBs. As yet, there are no human studies on exposure to environmental EDCs and adrenocortical function, although the importance of the role of glucocorticoids in immune suppression is known (section 5.4.1.2). The role of altered hormonal homeostasis in early life in the generation of disease in later adult life is a relatively new field of research (Marmot and Wadsworth, 1997) and is clearly of potential relevance to the endocrine disruptors debate. For example, the possible role of elevated glucocorticoid levels in fetal life in later development of diabetes has already been mentioned (Chapter 3, section 3.2.3). These areas undoubtedly need further investigation.

Chapter 6: Exposure of Selected Potential EDCs in Humans and Wildlife

6.1 Introduction

The availability of validated exposure data is a critical component for assessing the causal relationships between exposure to EDCs and health effects. Previous chapters examined the state of the science regarding potential effects of EDCs in wildlife and humans. This chapter focuses on exposure issues and analytical approaches and methodologies particularly relevant to EDCs, by using some illustrative case studies in wildlife and humans. It is not intended to evaluate all data published on exposures to potential EDCs. Knowledge about the magnitude or patterns of human and wildlife exposure to EDCs is limited. Current available exposure information has focused mainly on concentrations of POPs in Europe and North America. There is limited information on exposures in developing countries and on the less persistent EDCs. POPs have been transported all over the world, even in regions where they have never been used. There may be considerable redistribution of POPs from warmer to cooler climates (de March et al., 1998). Even though a number of POPs are no longer produced, they have remained in the environment for many decades and may continue to be unintentionally produced during some industrial processes. A large amount of data was compiled and evaluated for this chapter. These data are summarized in Annex I. Most of these data, however, were not generated specifically for assessing relationships between exposure to EDCs and adverse health outcomes. In only a few cases have such relationships been clearly established (see Chapters 4, 5, and 7).

Exposure studies aim to determine the nature and extent of contact with a chemical under different conditions and involve both external measurements (e.g., levels in air, water, food) and internal measurements (e.g., levels in blood, urine, breast milk). Generally, approaches include indirect and direct techniques, measurements of environmental or tissue concentrations, questionnaires, personal monitoring devices, biomarkers, and mathematical models (Paustenbach, 2000; IPCS, 2000). Some knowledge of the sources, fate, and transport of a chemical and its transformation or degradation in specific media and/or species is required. Emphasis is placed on assessing the magnitude, duration, and frequency of exposure as well as on estimating the number of individuals involved. Comprehensive exposure studies are very costly and are often constrained by limited resources and ethical considerations. Therefore, priorities must be set and the purpose must be clearly defined. Reasons for undertaking exposure assessments include epidemiology or field studies, status and trend determinations, and for risk assessment analysis or risk management purposes.

In this document, EDCs have been defined as "exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub) populations." The diversity of chemicals includes natural and synthetic hormones, phytoestrogens, pesticides, and a variety of industrial chemicals and by-products. This enormous diversity means that it is not possible to define a "typical" EDC, and each case must be carefully evaluated as to what chemical(s) to measure, in what matrix or biological tissue. In addition to their structural diversity, EDCs possess a range of different physicochemical characteristics. Some are persistent and lipophilic, sequestered in adipose tissue and secreted in milk, whereas others are hydrophilic and rapidly degraded and may only be present for short periods of time but at critical periods of development. Some complex mixtures (e.g., sewage waste treatment, industrial effluents) contain "hormonally active" components, but the specific chemical entity has not been fully characterized. The ubiquitous presence of natural hormones and plant estrogens poses difficult analytical issues because these natural EDCs may be more potent than environmental EDCs.

In general, exposure studies for EDCs are similar to those for other toxic chemicals. There are, however, some issues that require special emphasis, including the diversity of chemicals reported to be hormonally active, the timing and duration of exposure, and the general inadequacy of the information currently available on exposure to EDCs.

One of the most important issues that complicate assessing exposure to EDCs in both wildlife and humans is the level, timing, and duration of exposure relative to the developmental stage of the organism (see Chapter 2). Exposure during fixed time frames in development when programming of the endocrine system is occurring may result in permanent changes, whereas exposure during "nonprogramming" time periods may not result in any significant or detectable effect (see Chapter 3). For wildlife, critical periods of development may include *in utero* or *in ovo* exposures, exposure at different stages of the life cycle, or exposure at different stages of the

List of Abbreviations

AMAP Arctic Monitoring and Assessment Programme
APs Alkylphenols
APEs Alkylphenol ethoxylates
APEOs Alkylphenol polyethoxylates
BAF Bioaccumulation factor
CALUX Chemical-activated luciferase gene expression
DDE Dichlorodiphenyl dichloroethylene
DES Diethylstilbestrol
DDT Dichlorodiphenyl trichloroethane
d.w. Dry weight
E_2 17β-Estradiol
EDCs Endocrine-disrupting chemicals
ELISA Enzyme-linked immunosorbent assay
EO Ethoxy unit
ER Estrogen receptor (α and β isoforms)
HCB Hexachlorobenzene
IARC International Agency for Research on Cancer
IPCS International Programme on Chemical Safety
NHANES National Health and Nutrition Survey
NP Nonylphenol
NPEs Nonylphenol ethoxylates
NPECs Nonylphenol polyethoxycarboxylates
NRC National Research Council
OCs Organochlorines
OH-PCBs Hydroxylated polychlorinated biphenyls
PBDEs Polybrominated diphenylethers
PCBs Polychlorinated biphenyls
PCDDs Polychlorinated dibenzodioxins
PCDFs Polychlorinated dibenzofurans
POPs Persistent organic pollutants
QA/QC Quality assurance/quality control
SARs Structure–activity relationships
SCOOP Scientific Cooperation on Questions Relating to Food
SEPA Swedish Environmental Protection Agency
TBT Tributyl tin
TCDD 2,3,7,8-Tetrachlorodibenzyl-*p*-dioxin
TEFs Toxic equivalent factors
TEQs Toxic equivalent quotients
TIE Toxic identification evaluation approach
UNEP United Nations Environment Programme
USA United States of America
US EPA United States Environmental Protection Agency
WHO World Health Organization

reproductive cycle that may have a strong seasonal element. Different life stages may occur in different environments (e.g., in some insects the eggs develop in aquatic environments but adults are terrestrial) and thus are subject to different exposures. In certain wildlife species, pups exposed to biomagnified EDCs in the fat-rich mother's milk (in seals the fat content may exceed 70%) may consume much higher levels of contaminants per kilogram of body weight during the lactation period than during later life stages. It is evident that exposure to EDCs during the fetal or equivalent developmental phase in mammals, birds, and fish is of major importance (see Chapters 4 and 5). For example, exposure to PCDDs/PCDFs and dioxinlike PCBs has been linked to reproductive impairment and teratogenic effects in colonial, fish-eating birds of the Great Lakes (Bowerman et al., 1995; Helander et al., 1999; Tillitt et al., 1989, 1992) and to reproductive and immune dysfunction among Baltic seals (Bergman, 1999a). Exposure to NP during smoltification in Atlantic salmon (Fairchild et al., 1999) can cause adverse effects. In fish, periods of susceptibility occur during fin development that may be greatly influenced by retinoic acid exposure (Vandersea et al., 1998). Retinoic acid is a known vertebrate teratogen (La Clair et al., 1998) and has also been shown to be important to metamorphosis in frogs. PCBs are reported to interfere with retinoic acid activity (Burkhart et al., 1998) and may exert effects via this mechanism (Vandersea et al., 1998).

In humans, age at which exposure occurs (i.e., during certain developmental stages) is also critical to assessing potential effects of EDCs (see Chapter 5). *In utero*, neonatal, childhood, and puberty appear to be critical developmental periods potentially susceptible to interference by EDCs. For example, exposure to EDCs during brain development can have permanent effects, whereas similar exposures to a fully differentiated brain could have no detectable effects. It has been demonstrated that the effects of exposure to PCBs on neurobehavioral function in children will vary depending upon whether exposure occurs prenatally or postnatally (Jacobson and Jacobson, 2001). Both the severity and type of effect observed are affected by the dose and duration of PCB exposure. Recently, Longnecker et al. (2001) have shown that *in utero* exposure to DDT is associated with small-for-gestational-age babies at birth, whereas postnatal exposure has no detectable effects on child growth. Exposure data must be collected during critical periods of development in order to adequately assess the potential impacts of EDCs in both wildlife and humans. This is a difficult but crucial task and has broad implications for designing future EDC monitoring and exposure assessment studies.

Some data on the magnitude and temporal trends of global exposure to POPs are available, but often the data were collected, analyzed, or reported in different formats, making it difficult to compare data sets and evaluate new data. In general, environmental and tissue levels of certain chlorinated POPs (e.g., PCBs) have declined in some countries in response to regulations banning or phasing out these chemicals (UNEP, 2001), but they remain of concern in many countries and uncertainty still exist regarding future trends. In contrast to the chlorinated compounds, the levels of certain polybrominated compounds (e.g., PBDEs) appear to be increasing (Meironyté et al., 1999). As part of the global treaty on POPs, UNEP is initiating additional regional monitoring programs for potential EDCs (UNEP, 2001).

Most of the data on EDC exposure involve relatively highly exposed adult populations. Very few data are available on lower levels of exposures at different life stages, although some countries are starting to initiate exposure-monitoring programs in children and pregnant women (Needham and Sexton, 2000; Noren and Meironyté, 2000). Very little information is available for the less persistent EDCs (e.g., phthalates, APs), with the exception of TBT, where impacts and exposures have been evaluated in more detail. Thus, the questions remain: Where, when, how, and for whom are exposures to EDCs likely to occur in various regions of the world? What are the similarities in exposures between developing and developed countries for both humans and wildlife? What populations and groups are most vulnerable? In order to adequately address these questions, a global, coordinated, collaborative monitoring and research program on EDC exposure/response relationships is urgently needed.

6.2 General Exposure Issues

As mentioned previously, exposure issues and methodologies for EDCs are generally similar to those of other chemicals. This section briefly summarizes the following general exposure issues: sources of exposure, fate and transport in the environment, exposure pathways in various media, bioavailability, bioaccumulation and pharmacokinetics, and internal dose (see Figure 6.1).

6.2.1 Sources

The wide range of chemicals shown to possess hormonal activity has been noted previously. Some environmental EDCs may be released into the environment intentionally (e.g., pesticides), but for most environmental contaminants release is unintentional. Unintentional release of chemicals can occur throughout part or all of the chemical's life cycle (e.g., manufacturing, use, disposal). "Dioxinlike" chemicals (e.g., PCDDs/PCDFs) are formed unintentionally as by-products in a variety of industrial and combustion processes (Fara, 1999; Tobin, 1986). Leakage from landfill areas and distribution via sewage sludge are also sources of exposure (Daughton et al., 1999). Exposure to naturally occurring EDCs such as the phyto- and fungal estrogens, which are important components of some human and wildlife diets, occurs globally. The isoflavonoid phytoestrogens are found in soy and legumes, the lignanes in grains and many fruits and vegetables, and the coumestans in clover and alfalfa. All have relatively short half-lives in humans, and metabolites can be detected in urine and feces. Phytoestrogens have also been detected in effluents from pulp mills, resulting in reproductive effects in certain fish species (see Chapter 4). The extent of exposure to natural EDCs will vary dramatically among species, individuals, and localities.

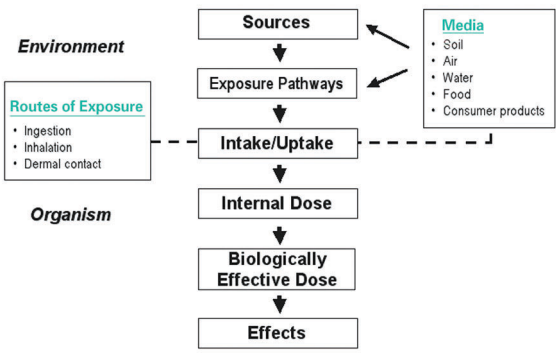


Figure 6.1 - Pathways of exposure and effects.

6.2.2 Exposure Pathways

The range of different physicochemical characteristics possessed by EDCs means that these chemicals will degrade and behave in different ways in the environment, impacting exposure routes for both humans and wildlife. The main abiotic factors that enhance the degradation processes are elevated temperature, increased sunlight, and aerobic conditions, so degradation rates might be expected to be faster in warmer, sunnier parts of the world. Other processes (e.g., hydrolysis, oxidation, radical and photochemical reactions) may also transform chemicals in the environment. In contrast, some suspected EDCs (e.g., POPs) do not readily degrade in the environment and may accumulate in some compartments (e.g., in sediment) or be transported long distances from their original sources.

Exposure can occur via air, water, soil, sediment, and food and consumer products. The chemical may then enter the organism by ingestion, inhalation, or skin contact (including via the gills) across cell membranes and then be absorbed into the bloodstream (Crosby, 1998). The contribution of various exposure pathways vary among species and life stages and are briefly summarized below.

6.2.2.1 Air. The air concentration of any chemical is dependent on its inherent and relative volatility when in contact with water, vegetation, and soils. Meteorological conditions (e.g., temperature, wind speed, humidity) will influence the air concentration and should be considered if assessing exposure regionally or globally. Less volatile chemicals may also be present in particulate matter in air. Semivolatile substances, which include the majority of known EDCs, may be more or less strongly bound to particulate matter in the air, thus affecting possible absorption into the bloodstream and uptake via the gastrointestinal tract. Other EDCs in air may be deposited in terrestrial systems on leaves, needles, grass, and soil (Jones et al., 1994) and in aquatic systems, where they may enter the food chain (Stapleton et al., 2001). The air concentrations of EDCs and their distribution between particulate matter and the gas phase influence the capacity for long-range transport and thus indirectly influence exposure via marine, limnic, and terrestrial systems. EDCs of potential importance for exposure via air consist of those with low masses, such as persistent halogenated compounds (e.g., lindane) and nonhalogenated mono-, bi-, or polycyclic aromatic hydrocarbons, phenols, and phthalate esters. Calculation of the absorbed dose of an EDC from air requires experimental data or estimates from models. It is possible to measure, or calculate, the inhaled dose in humans if the air concentrations, breathing rate, and absorption efficiency are known (Paustenbach, 2000). Although a number of potential EDCs are regularly monitored in air as part of national air monitoring programs, such data are rarely collected to specifically assess exposure to EDCs in wildlife or humans.

6.2.2.2 Water. Water both is the surrounding medium of a large number of water-breathing species (e.g., fish, aquatic invertebrates) and is consumed by humans and terrestrial species. A variety of pesticides, industrial chemicals, and natural hormones have been detected in surface waters. Chemicals may be dissolved in water and/or bound to particulate matter. In water-dwelling species, uptake can occur through direct contact via the gills or as they feed. Bioconcentration of semipersistent and persistent organic chemicals depends on the equilibrium partitioning between body lipids and the ambient water, with the gill surface playing an important role (MacKay and Fraser, 2000). Fish and/or mussels have been shown to accumulate NPs (Larsson et al., 1999; Lye et al., 1999; Wahlberg et al., 1990), halogenated phenols (Asplund et al., 1999), and ethinyl estradiol (Larsson et al., 1999) from surrounding waters.

Drinking water is a potential source for human exposure to EDCs, although it is not a major exposure pathway unless unusual contamination has occurred. In developed countries, drinking water is generally treated to remove microbial contamination, suspended particulate matter, and some hazardous chemicals (e.g., pesticides, aromatic hydrocarbons). In some instances, other chemical contaminants are introduced as part of the water treatment process (IPCS, 1999). In developing countries, drinking water is not generally treated, and it is often contaminated with industrial and naturally occurring chemicals. Models are available to calculate the absorbed dose of a contaminant from water (IPCS, 2000). Drinking water is not regarded as a major source for exposure to persistent lipophilic chemicals (Liem et al., 2000).

6.2.2.3 Soil. A number of potential EDCs (e.g., PCBs, dioxins, PBDEs) have been detected in soils and/or sewage sludge in different parts of the world (Lega et al., 1997; Hale, 2001; Kocan et al., 2001; Stevens et al., 2001). For certain wildlife species (e.g., worms, snails, insects) that live in close contact with the soil, this may be a major route of exposure. These organisms are a part of the food chain for certain birds and terrestrial animals. Farm animals may be exposed to contaminated soil through grazing and thus contribute to human exposure via this food chain pathway.

6.2.2.4 Sediment. Sediment may be a pathway of exposure for certain wildlife species living in close contact with or in sediments for all or parts of their life cycle. Some chemicals will partition to particulate matter suspended in water, which may be deposited and accumulated in sediments. The continuous fallout and redeposition of atmospheric particulate matter to which lipophilic substances may be bound also increase exposure via this pathway. Data on PCDDs/PCDFs, PCBs, and some brominated flame retardants in sediments from European estuaries are available (Sellström et al., 1999a; van Zeil, 1997; Olsson et al., 2000c). Less persistent chemicals such as alkyl ethoxylates and NPs have also been reported in sediments (Bennett et al., 1998; Lye et al., 1999), as have estrogenic and androgenic steroids (Thomas et al., 2001). Human exposure via this route is low and restricted to consumption of bottom-feeding organisms.

6.2.2.5 Food. Ingestion of EDCs and potential EDCs via food intake is generally considered the major exposure route both for humans and most wildlife and may lead to bioaccumulation and biomagnification. The contribution of dietary exposure will vary as a function of dietary preferences, position in the food chain, and species and quantities consumed. Persistent, lipophilic organic pollutants often bioaccumulate in species at the top levels of the food chain. Fish-eating birds and marine mammals have been found to have concentrations of POPs many times higher than those found in fish on which they feed, or compared with levels in the surrounding waters. Sometimes the levels can be elevated by a factor of hundreds or millions (SEPA, 2001). For example, in Baltic gray seals, total DDT and PCB concentrations in fatty tissues averaged 100 times higher that those found in their major food source, herring (Bignert et al., 1998c). Polar bears, which constitute the end of the marine food webs of the Arctic, have total PCB levels 100 times higher than levels in Scandinavian women (Henriksen et al., 2001a). The high PCB levels in these top predators are due to bioaccumulation of very specific PCB congeners.

In general, marine mammals, predatory and piscivorous birds and predatory fish have higher concentrations of POPs than do terrestrial wildlife and wildlife at lower trophic levels (Jansson et al.,

1993). It is among these animals that effects such as reproductive failure, teratogenic effects, and eggshell thinning have been observed (see Chapter 4). In certain wildlife species, pups exposed to EDCs in the fat of mother's milk may consume much higher levels of contaminants per kilogram of body weight during the lactation period than during later life stages. Humans tend to consume both plant and animal food, and because food choices vary among population cultures and geographic regions, exposure to potential EDCs will differ considerably from one person to another. Humans relying on contaminated species for subsistence foods have been shown to accumulate higher levels of POPs (Borrell et al., 1993; Hansen et al., 1998; Lindström et al., 1999). Some of the highest concentrations of POPs have been observed in populations such as the native Inuits in northern Canada and Greenland (Ayotte et al., 1997; Hansen et al., 1998) and women from the Faroe Islands (Fängström et al., 2002). Similarly, heavy consumers of fatty fish from contaminated have been shown to have higher residues of persistent lipophilic chemicals (Jacobson et al., 1996; Svensson et al., 1991, 1995). For infants, breast milk may be a major source of exposure to EDCs, coinciding with a sensitive time for growth and development. Very little is known about EDC levels in bottled baby milk or infant foods.

Human exposure to chemicals via food can be assessed directly through chemical analysis of food items in duplicate diet studies, or indirectly through market basket/total diet surveys (e.g., food diaries, food frequency questionnaires). Total diet surveys have been carried out for many years in a number of countries to ensure the safety of national food supplies (FAO/WHO, 1995). Methods for estimating exposure to EDCs via food must be adapted to specific situations because diets vary greatly in different countries and different subpopulations.

Human dietary exposure to persistent lipophilic chemicals has been relatively well studied. An extensive assessment of the dietary intake of PCDDs/PCDFs and dioxinlike PCBs in 10 countries was recently published by the European Union (SCOOP, 2000). Other studies (Darnerud et al., 2000) indicate that in developed countries, dairy products, meat, and fish and fish products are the most important sources of exposure, with proportions dependent on the source of the food item (e.g., fish from the Baltic Sea) or the exposure situation (e.g., cow's milk from The Netherlands) (Liem et al., 2000).

6.2.2.6 Consumer products. For humans, a wide range of consumer products (e.g., cleaning products, personal products, cosmetics, garden chemicals) provide pathways of exposure via inhalation, ingestion, and dermal contact. Of particular concern is the potential for phthalate ester exposure in young children orally by chewing on toys and teething rings (Steiner et al., 1999).

6.2.3 Intake and Uptake

Intake is associated with inhalation and ingestion routes of exposure, whereas uptake is associated with dermal contact. If there is no active transport across cell membranes, absorption is dependent on the concentration gradient and ability of the chemical to cross cell membranes. Chemicals with molecular masses up to almost 1,000 Da have been shown to be bioavailable and to be transferred over biological membranes (El Dareer et al., 1987). Most environmental potential EDCs have masses in the range of 200–600 Da. If bioavailable, the internal dose is similar to the concentration in the medium (usually blood) in the vicinity of the site for absorption or uptake. The internal dose may be much higher locally due to specific binding properties of the EDCs or their metabolites in certain cell types or tissues, because some EDCs may bind to hormone transport proteins or cellular receptors (Lans et al., 1993; Lund et al., 1988; Brouwer et al., 1998; Poellinger, 2000). Active transport mechanisms may also play a role in the absorption of EDCs (Tsuji and Tomai, 1996).

6.2.4 Internal Dose and Pharmacokinetics

The internal dose is the amount of the chemical absorbed and able to undergo metabolism, transport, storage, or elimination. Principal matrices for determination of the internal dose are blood, breast milk, adipose tissue/blubber, and muscle tissue. It is possible to obtain all these from wildlife, but they require invasive sampling except for milk, which presents particular practical problems with sampling. For humans, blood and mothers' milk are relatively easy to sample, but ethical and social considerations must be taken into account. Internal exposure data are more difficult to obtain for substances with short half-lives unless they are present at steady-state levels in the tissue. For readily metabolized compounds with characterized metabolites, exposure may be estimated from the concentration of metabolites in excretion products. The nature of the exposure and the time course of metabolism, transport, storage, and elimination of a chemical have important implications for when to collect samples. Measurements of the internal dose have a number of advantages, including demonstration that exposure has occurred, integration over all exposure routes, and inclusion of dosing from internal sources (e.g., remobilization of lipid-soluble EDCs). Limitations include lack of information about sources and routes of exposure, the timing issues mentioned above, and the general lack of background information for comparison.

The pharmacokinetics of chemicals plays a major role in determining exposure (van Birgelen and Van den Berg, 2000). EDCs can exert hormonal activities either because of their intrinsic activity or through their metabolic products (see Chapters 4 and 5). Persistent lipophilic chemicals, such as PCBs, may form both persistent metabolites and less persistent phenolic type metabolites (Letcher et al., 2000). Several aromatic compounds are known to form hormonally active metabolites, such as DDE from DDT (Metcalf, 1973), methylsulfonyl-DDE from DDE (Lund et al., 1988), and polychlorobiphenylols (OH-PCBs) from PCBs (Letcher et al., 2000, Sundström et al., 1976). A number of PBDEs can be biotransformed to OH-PBDEs (Örn and Klasson Wehler, 1998), and alkenylphenol ethoxylates to APs (Sharpe et al., 1995; Nimrod and Benson, 1996). EDCs and their metabolites are transported within organisms by the same routes and mechanisms as all xenobiotics. The uptake of chemicals through the blood–brain barrier across the placenta is of great importance and is influenced by the structure and polarity of the chemical. For example, it appears that phenolic compounds are more easily transferred to the fetus than are neutral compounds (Sauer, 2000; Meironyté Guvenius, 2002). Examples of specific retention of certain EDCs in tissues other than lipid include the liver (PCDD/PCDF and other dioxinlike chemicals) (Birnbaum and Tuomisto, 2000), lung (PCBs, methylsulfones) (Brandt et al., 1985; Lund et al., 1985), blood retention of phenolic substances (Bergman et al., 1994; Sandau et al., 2000; Sjödin et al., 2000), and binding of a DDE methyl sulfone to the adrenal cortex (Lund et al., 1988).

6.3 Case Studies

In this section, several case studies are summarized for both wildlife and humans to illustrate the range and types of exposure information available for selected potential EDCs in different parts of the world.

Only general conclusions are discussed; the data supporting these conclusions are compiled in Annex I.

6.3.1 Wildlife Exposures

The following case studies were chosen to illustrate the diversity of routes of exposures and species affected and the importance of biomagnification of persistent EDCs through the food chain. Exposure information is summarized for concentrations in the environment and in biota and, where available, for temporal trends.

6.3.1.1 Persistent lipophilic EDCs. As mentioned above, the most comprehensive data sets available focus on certain POPs such as DDT and PCBs. Selected exposure data are summarized here from three diverse regions of the world representing different ecosystems: 1) the Baltic Sea, a marine enclosure; 2) the Great Lakes in the USA, a freshwater lake system; and 3) the Arctic, a relatively harsh remote environment. Table 6.1 lists examples of persistent EDCs, which have impacted these regions, and species that have been affected and/or studied.

6.3.1.1.1 The Baltic Sea.

ENVIRONMENT. The Baltic Sea is one of the marine areas that has been most seriously polluted by DDT and PCBs, due to the presence of highly industrialized communities within its drainage areas. This ecosystem is located in the temperate zone of the world, with clear seasonal variations. In the north, the surface is generally covered by ice for some months, whereas the southern areas are rarely ice covered. The water is brackish; the tidal movement is a few centimeters in the south and imperceptible in the north. Because the Baltic Sea is a marine enclosure, the importance of dilution processes by exchange of clean water from the North Atlantic is small. Furthermore, the water circulation within the Baltic is reduced because of a strong halocline, with higher salinity in bottom water and lower salinity in surface water. The surface water temperature has higher seasonal variations than do most other marine waters. There are comparatively few species in the Baltic Sea. The biological community comprises a mixture of marine species (herring, cod, seals, etc.) as well as freshwater species (perch, pike, etc.). For the top consumers, the fatty herring is an important food item, and its role in bioaccumulation in marine birds and mammals (as well as in humans) explains some of the detrimental effects found in the Baltic Sea ecosystem.

Potential endocrine-mediated adverse effects have been observed in a number of species in the Baltic Sea. Examples include an increased prevalence of female salmon producing offspring with a low survival rate (Johansson and Ahlborg, 1994); lowered reproductive capacity and eggshell thinning in white-tailed sea eagles (Stjernberg et al., 1990; Helander et al., 1998, 1999b; Odsjö et al., 1977), razor bill (Andersson et al., 1988), and guillemot (Bignert et al., 1995), and immune and reproductive impairment in marine mammals (Bergman et al., 1985; Simms and Ross, 2001).

CONCENTRATION DATA. The concentrations of various EDC compounds in some selected matrices in representative Baltic species are summarized in Annex I (Tables 1–10). Extensive data are provided for herring and salmon, because both are important food items for consumers at the top of the food chain, including humans. These summary tables illustrate the difficulties encountered when trying to compare different studies. In some instances, sampling was carried out at different times of the year, organisms were sampled at different stages in the life cycle, samples were collected and analyzed using different methods, and results were analyzed and reported using different formats. In order to make the data comparable to some degree, data are provided in these tables on a lipid weight basis in micrograms per gram, which required estimation of fat content in some matrices and species. Concentrations of DDT and PCBs were highest in white-tailed sea eagle eggs sampled during the 1960s and 1970s, and although these levels decreased during the 1980s and 1990s, concentrations remain high compared with species lower in the food web. Figures 6.2 and 6.3 summarize data on total PCB and DDT concentrations for species at several trophic levels for the periods 1969–1972 and 1988–1998.

TEMPORAL TRENDS. Monitoring studies of the Baltic environment have covered a number of species, including terrestrial, freshwater, and marine biota (Bignert et al., 1998a). Interestingly, annual changes over time have been shown to be very similar for the compounds measured, whether the organisms were from terrestrial, freshwater, or marine environments (Bignert et al., 1998b, 1998c; Olsson et al., 1997, 1998). However, no significant decrease of dioxins was observed in guillemot eggs and herring from the Baltic over the last 10 years, while DDE and HCH levels continued to decrease (Olsson et al., 2002).

Data on temporal trends are summarized in Annex I (Figures 1–7). Total PCB concentrations of guillemot eggs declined from around 300 µg/g lipid in 1969 to around 30 µg/g lipid in 1999. Similar trends are shown for DDTs, HCBs, and TEQs of PCDDs/PCDFs in guillemot eggs (see Annex I). Except for PBDEs and chlordanes, concentrations have decreased since the beginning of the 1970s (Bignert et al., 1998a; de Wit et al., 1994; Odsjö et al., 1997). The temporal trends for α-HCH and γ-HCH (lindane) as determined in herring showed similar decreases. Decreasing concentrations of toxaphene during 1974–1989 in the environment have also been reported (Wideqvist et al., 1993). For PBDEs, temporal trends show a different pattern. Concentrations of PBDEs in guillemot eggs increased from the late 1970s to the late 1980s and

Table 6.1 - Examples of Persistent EDCs in Wildlife in Three Geographic Regions

	Baltic Sea	Great Lakes (USA)	Arctic
Environment	Marine enclosure, little clean water dilution, strong halocline; temperate climate	Connected freshwater lakes; temperate climate	Dramatic seasonal differences; polar seas and lakes covered by ice for much of the year
Chemicals	DDT, PCBs, HCB, PCDDs, PCDFs, PBDEs, HCHs	DDT, PCBs, PCDDs, PCDFs, PBDEs	DDT, PCBs (including OH-PCBs), PCDDs, PCDFs
Sources	Highly industrialized communities within drainage areas	Some areas of shoreline highly industrialized	Highly industrialized areas on periphery; long-range transport for some pollutants
Examples of species affected			
Fish	Salmon	Lake trout, chinook salmon	Arctic char
Birds	White-tailed sea eagle, guillemot, razorbill	Herring gulls, Forster's terns, double-crested cormorants	Glaucous gull, thick-billed murre, puffin, white-tailed eagle
Mammals	Gray seal, otter, mink	Mink	Mink, otter, polar bear, ringed seal

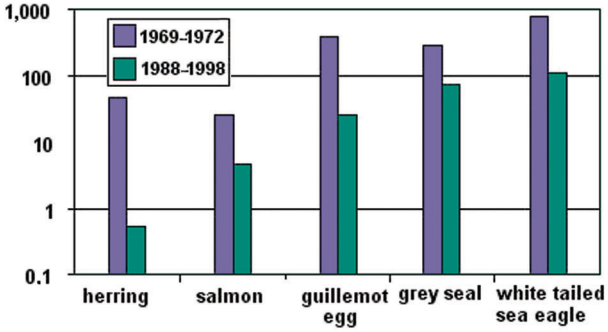


Figure 6.2 - Representative concentrations of total DDT (µg/g lipid weight) in selected Baltic species from samples collected during 1969–1972 and 1988–1998 (for more details see Annex I, Table 1). All concentrations are log transformed.

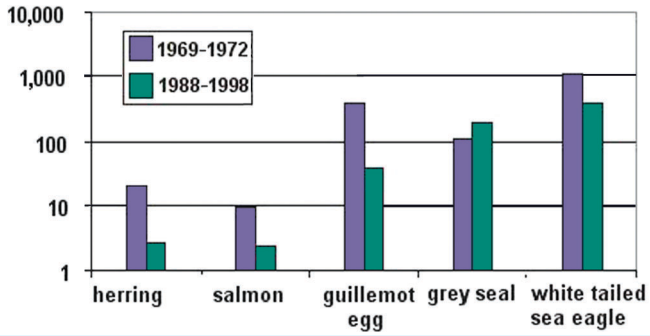


Figure 6.3 - Representative concentrations of total PCB (µg/g lipid weight) in selected Baltic species from samples collected during 1969–1972 and 1988–1998 (for more details see Annex I, Table 2). All concentrations are log transformed.

then declined (Kierkegaard et al., 1999; Moilanen et al., 1982; Sellström et al., 1999b).

The decreasing concentrations of OCs mentioned above have been followed by a concurrent improvement in reproductive capability for white-tailed sea eagle (Helander et al., 1999), in eggshell thickness in guillemot (Bignert et al., 1995), and in increasing populations of otter (Roos et al., 2001), mink (Bergman et al., 1992), gray seal (Helander et al., 1999), harbor seal (Helander et al., 1992), and ringed seal (Härkönen et al., 1999). Although reproductive health has improved in seals, some problems remain among gray seal (Bergman, 1999a) and ringed seal (Mattson et al., 1995) (See Chapter 4).

6.3.1.1.2 The Great Lakes.

ENVIRONMENT. The Great Lakes comprise a large system of connected freshwater lakes lying mainly along the USA–Canada border. Water flow is from Lake Superior (with fairly oligotrophic water) in the northwest through Lake Michigan (with heavy industries) just south of Lake Superior, then through Lake Huron, Lake Erie, and Lake Ontario (the latter three lakes are highly eutrophic and polluted by municipal, agricultural, and industrial effluents) to the St. Lawrence River. Numerous large cities are located along the shores of these lakes, many with heavy industries, including producers of pesticides and OC compounds. This has led to widespread contamination by such substances from air, the watersheds, and industries and urban areas located along the shoreline. Lake Superior is considered to be less contaminated than the other lakes and less influenced by local industries. Most of the lakes have been highly contaminated with PCBs, DDT, PCDDs/PCDFs (particularly TCDD), and a number of OC pesticides.

Potential endocrine-mediated adverse effects have been observed among species in the Great Lakes area (see Chapter 4). These include poor reproduction in fish, such as Chinook salmon (*Oncorhynchus tshawytscha*) and lake trout (*Salvelinus namaycush*), and birds including herring gulls (*Larus argentatus*) (Fox et al., 1998), Forster's terns (*Sterna forsteri*), double-crested cormorants, Caspian terns (*Sterna caspia*) (Gilbertson, 1989), and bald eagles (*Haliaeetus leucocephalus*; Best et al., 1994).

CONCENTRATION DATA. Concentrations of DDT, PCBs, and PCDDs/PCDFs in representative species for the Great Lakes are summarized in Annex I (Tables 11 and 12; Figures 8 and 9). Data are taken from studies carried out in the 1980s and 1990s from which sufficient QA/QC could be obtained. PCB levels are reported as total PCB or the sum of varying numbers of PCB congeners. TCDD TEQs were calculated from reported concentrations of PCDDs/PCDFs. Similar to the Baltic Sea observations, concentrations of DDT/DDE, PCBs, and PCDD/PCDFs were highest in herring gull bird eggs sampled in the early 1970s. Although levels decreased during the 1980s and 1990s, the concentrations remain higher in herring gulls, reflecting their position at the top of the food web.

TEMPORAL TRENDS. The Canadian Wildlife Service has monitored herring gull eggs annually from 16 sites covering all five Great Lakes for levels of DDT, PCBs, and dioxins (see Annex I, Figures 10–13). An analysis of the temporal trends for PCBs and DDE from 1974 to 1995 indicates that concentrations of these have generally declined in all the Great Lakes (Pekarik et al., 1998). However, temporal trends vary among individual sites. For example, in Lakes Michigan, Huron, Erie, and Ontario, PCB levels have continued to decline at the same rate from 1974–1975, whereas the rate of decline for PCB concentrations in western Lake Ontario slowed between 1987 and 1995 and ceased after the mid-1980s in Lake Superior. For Lake Michigan (Green Bay area), PCB concentrations have shown no significant temporal trend since 1976. OC concentrations have also declined significantly in double-crested cormorant eggs between 1970 and 1995 in Lakes Ontario, Superior, and Huron but not in Lake Erie (Ryckman et al., 1998). Lake trout from Lakes Michigan, Ontario, Huron, and Superior have been monitored for PCB and DDT concentrations from the 1970s to the 1990s. For Lake Ontario, data also exist for dioxinlike compounds. Concentrations for all these compounds have generally declined from the 1970s to the 1990s (Borgmann et al., 1991; De Vault et al., 1986; Huestis et al., 1996, 1997). In contrast, PBDE levels have continuously increased 20–60 times from 1981 to 1999 in herring gull eggs from Lake Huron (Channel/Shelter Island), Lake Michigan (Gull Island), and Lake Ontario (Snake Island). A continuous increase in PBDE concentrations has also been observed in Lake Ontario Lake trout caught during 1978–1998 (see Annex I, Figure 13) (Luross et al., 2000). Low concentrations of PBDEs have been reported in Lake Michigan lake trout (Manchester-Neesvig et al., 2001).

6.3.1.1.3 The Arctic region.

ENVIRONMENT. The Arctic region has a unique cold climate with extreme seasonality in light and productivity. Lakes and large sea areas are covered by ice much of the year. Species diversity is low, and food webs are relatively simple but include third-level carnivores (polar bears). Primary production is higher in aquatic ecosystems than in terrestrial ecosystems. The ecological systems are generally oligotrophic. Lipids play an important role as an energy source in the Arctic food web, which leads to high bioaccumulation and biomagnification of lipophilic endocrine disruptors in upper trophic level animals. The extreme seasonality of the Arctic also

leads to large fluctuations in the lipid stores of many organisms because of the need to store fat during the short productive season and consumption of stored fat when food is scarce. This in turn can lead to large fluctuations in blood concentrations of EDCs, with consequent redistribution in tissues, making it difficult to estimate dose–response relationships in some species.

Since 1981, the AMAP has monitored levels of certain environmental pollutants (de March et al., 1998). Concentrations of DDT, PCB, HCH, HCBs, and PCDD/PCDFs are summarized in Annex I (Tables 13–15; Figures 14–18) for representative species in the Arctic. These data extracted from AMAP studies (de March et al., 1998) reflect the highest and lowest concentrations of these chemicals measured anywhere in the Arctic. PCB levels are given as total PCB or the sum of varying numbers of congeners. TEQs are based on reported values. Data represent studies carried out in the 1990s where sufficient QA/QC could be obtained. Several studies indicate higher concentrations of PCB and DDT in certain parts of the Arctic (e.g., Russia), but the database is incomplete and further monitoring data is needed.

Concentrations of POPs in Arctic species reflect their position in the food web. For example, eggs of top prey species such as white-tailed sea eagles had higher concentrations than did species lower in the food web. Studies of Canadian seabirds have shown that eiders, which overwinter in contaminated waters, have a higher contaminant load than do birds overwintering in clean waters. In mammals, concentrations in marine species are higher than in terrestrial species and highest in top predators at the end of long food chains (e.g., polar bears) (de March et al., 1998). Higher OH-PCB concentrations than PCB concentrations were found in the blood of polar bears (Sandau, 2000).

TEMPORAL TRENDS. Monitoring in Arctic biota from Canada, Greenland, Norway, and Finland has been limited to small and infrequently collected samples (2–4 times over 25 years), making it difficult to determine temporal trends. Furthermore, high intrasite variability and changes in analytical methodology have made temporal comparison problematic. Generally speaking, limited temporal trend data indicate declining concentrations of PCB and DDT in the Arctic. Less is known about the temporal trends of many other persistent compounds, including HCHs, HCB, chlordane, toxaphene, dieldrin, and PCDDs/PCDFs.

Annual collection and analyses in pike in Lake Storvindeln (Annex I, Figure 17) and Arctic char in Lake Abiskojaure (Annex I, Figure 18) in subarctic Sweden for the past 20–30 years have provided some of the strongest evidence for declining levels of DDTs and PCBs northern Scandinavia (Bignert et al., 1998a). A sudden decline occurred soon after measures to reduce the discharges of DDT and PCB were implemented in the early and middle 1970s (Bignert et al., 1995, 1998a; Olsson et al., 1986). Since that time, the annual decline of DDT and PCB concentrations (3–8% a year) has continued (Bignert et al., 1998a).

6.3.1.1.4 Global distribution of DDT and PCB compounds in marine mammals. Marine mammals (e.g., harbor seals) represent species at the top of the food chain, and considerable monitoring data have been collected on tissue levels of POPs from various marine mammals in different parts of the world (see Annex I, Table 16). Data on polar bears are also included because these animals prey on seals in the Arctic. Figure 6.4 illustrates the range of PCB and DDT levels in fish-eating marine mammals from various parts of the world. Examples of effects of PCB and DDT contamination in marine mammals include low reproductive success and declining populations of harbor seals in the Dutch Wadden Sea (Reijnders, 1980, 1986, 1990), premature pupping in California sea lions (Gilmartin et al., 1976), and immune and reproductive impairment in Baltic ringed and gray seals (Bergman and Olsson, 1985; Roos et al., 1998).

Factors that affect tissue concentrations include species differences, food, age and sex differences, seasonal differences, and differences in the chemical analytical methods. Overall, the available data gathered indicate worldwide occurrence of POPs, including in the remote areas of the world. These data are scattered and spread over a large number of species, collection years, habitats,

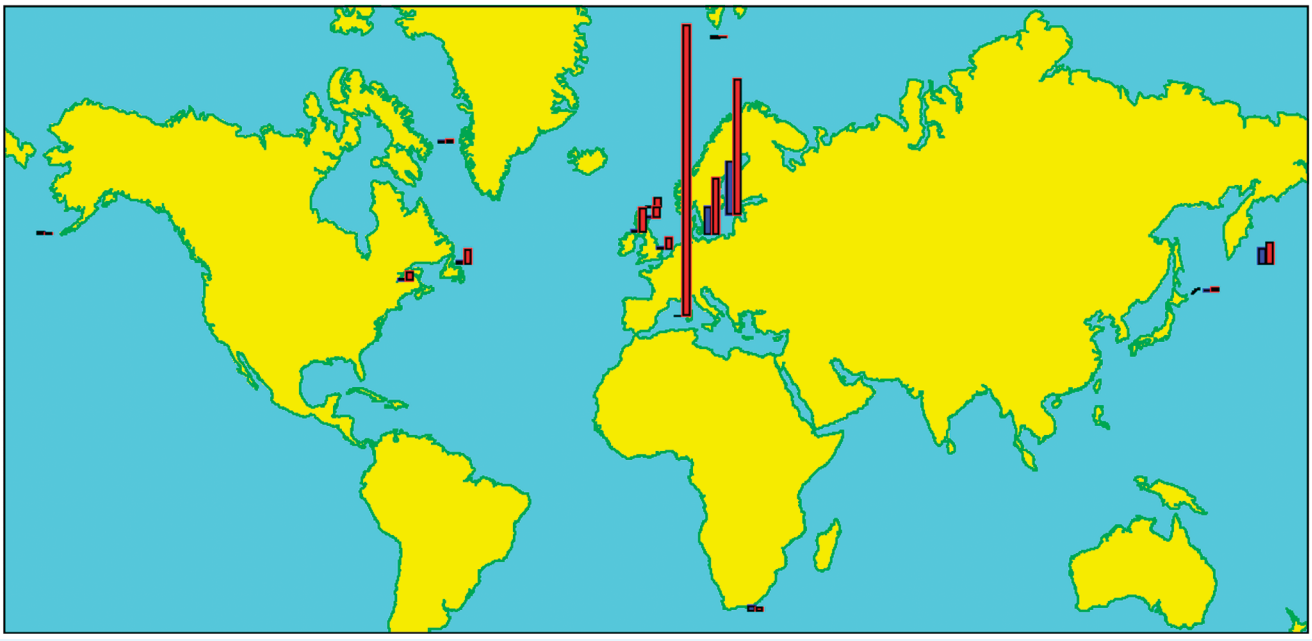


Figure 6.4 - Concentration range of total DDT (blue bars) and total PCB (red bars) (µg/g lipid weight) in marine fish-eating mammals from various parts of the world. Highest bar = 300 µg/g lipid weight (for further details, see Annex I, Table 16).

seasons, and chemical analysis methodologies, making it difficult to compare data and assess temporal and geographical trends.

6.3.1.2 TBT. TBT is found in antifouling paints applied to hulls of ships. TBT degrades in the environment to the less active di- and monobutyl tins. Studies on TBT concentrations in the environment and in biota have been carried out many locations throughout the world, including coastal areas around Europe, Asia, North America, and Australia as well as open ocean areas (Guruge et al., 1996, 1997; Iwata et al., 1995; Kannan et al., 1995a, 1995b, 1996, 1997; Kannan and Falandysz, 1997; Kim et al., 1996a, 1996b, 1996c; Takahashi et al., 1997, 1999; Tanabe et al., 1998, Muir et al., 2002). Coastal areas are affected by previous and current use of TBT on boats and fishing equipment near harbors and marinas. Sediments in particular seem to be reservoirs for TBT even after use has stopped, leading to continued exposure. Open ocean areas are exposed to TBT from large vessels that continue to use TBT on the their hulls. TBT has been reported to cause imposex (induction of a penis in females) in marine gastropods at very low concentrations and shell abnormalities in some species of bivalve mollusks (see Chapter 4).

6.3.1.2.1 Concentrations in the environment. Concentrations of TBT have been determined in water column and sediment samples from freshwater, estuaries, seawater, marinas, and harbors (see Annex I).

6.3.1.2.2 Concentrations in biota. Species analyzed for TBT include invertebrates, fish, eels, birds, and marine mammals. Analysis of TBT levels in fish and mammals has only been carried out since the mid-1990s. Higher concentrations are found in comparable matrices collected near coasts compared with those collected from the open ocean. For the Pacific Ocean area, highest concentrations are found in fish around Japan and Australia, with lower levels around developing nations such as India, Bangladesh, Thailand, Indonesia, and Vietnam. Concentrations in fish from the U.S. coast, Italy, and the Baltic Sea are fairly similar to one another and to concentrations in Japan.

Concentrations of TBT in various species are highly variable, because of differences in external exposure and metabolism (Annex I, Table 17). This is exemplified by monitoring studies of Otsuchi Bay on the Pacific Coast of northern Japan (Tanabe, 1998; Takahashi et al., 1999). Levels are generally low in invertebrates and fish compared with marine mammals. Cormorants from Lake Biwa, Japan, had total mono-, di-, and TBT concentrations of 140–1,000 ng/g wet weight in liver (Guruge et al., 1996). Diverse species of seabirds from around the Japanese and Korean coast had TBT concentrations ranging from nondetectable to 500 ng/g wet weight in liver, whereas oceanic birds had concentrations ranging from nondetectable to 29 ng/g wet weight in kidney (Guruge et al., 1997). Along the Polish coast of the Baltic Sea, several species of seabirds had TBT concentrations of 35–4,600 ng/g wet weight in liver (Kannan et al., 1997).

In marine mammals (primarily cetaceans and pinnipeds), a similar geographic trend is seen as for fish. Liver tissue samples from various species of cetaceans from the Japanese coast had 230–10,200 ng/g wet weight; from China, 350–1,200 ng/g wet weight; and from the Philippines and India, 40–200 ng/g wet weight (Tanabe et al., 1998). Liver tissue samples from several cetaceans from the U.S. Atlantic coast had 80–11,300 ng/g wet weight (Kannan et al., 1997). Bottlenose dolphins from the Italian coast had 1,200–2,200 ng/g wet weight, and neonatal harbor porpoise from the Baltic Sea had 18–27 ng/g wet weight in liver (Kannan and Falandysz, 1997). Liver tissue sample from pinnipeds from Japan had TBT concentrations of 50–320 ng/g wet weight (Tanabe et al., 1998), whereas along the Alaskan coast, concentrations were 2–24 ng/g wet weight in liver (Kim et al., 1996a).

6.3.1.2.3 Temporal trends. Because of its high toxicity to marine invertebrates at low water concentrations, many countries restricted TBT use during the 1980s. In France, restrictions were imposed in 1982, and over the period 1982–1985, water and oyster concentrations of TBT dropped 5–10 times, resulting in decreased oyster shell malformations and increased populations (Alzieu, 1991). Use restrictions have also led to reductions in concentrations in various media (water, sediment) and organisms (oysters, mussels, dog whelk) from Japan, the USA, and the United Kingdom, with concomitant increases in oyster shell growth and decreased imposex in dog whelk (Harino et al., 1999; Miller et al., 1999; Valkirs et al., 1991; Waite et al., 1991). Where exposure to TBT had caused irreversible imposex in dog whelks, there was a time lag in population recovery (Miller et al., 1999). No temporal trend data are available for fish, birds, or marine mammals. In Germany, restriction of TBT in 1989 resulted in decreasing TBT concentrations in freshwater fish from several German rivers. However, mussel and eelpout from the Wadden Sea (The Netherlands) showed no obvious decline in TBT concentrations over the period 1985–1998 (UBA, 2001a).

6.3.1.3 APs, APEs, and their degradation products.

6.3.1.3.1 Sources and fate of APEs. APEs are high-volume chemicals that have been used for more than 40 years as detergents, emulsifiers, wetting agents, and dispersing agents. APE-containing products are used in many sectors, including textile processing, pulp and paper processing, paints, resins and protective coatings, oil and gas recovery, steel manufacturing, pest control products, and power generation. Certain APEs are also used in a wide range of consumer products, including cosmetics, cleaners, and paints, and in a variety of applications. It is therefore not surprising that they have been widely detected in effluents and the environment.

APEs with more than eight ethoxy (EO) units (most common commercial products) are readily degraded in effluent treatment system with >92% efficiency (Kvestak and Ahel, 1994; Kubek and Naylor, 1990; Naylor et al.,1992; Brunner et al., 1988; Giger et al., 1987). The primary products remaining in effluents and sludge after treatment are APEs with low EO chain lengths, APEOs (AP1, 2EO), AP polyethoxycarboxylates (AP1, 2EC), as well as APs, which may ultimately be mineralized to CO_2 (Ahel et al., 1994; Ball et al., 1989; Yoshimura, 1986; Giger et al., 1984; Lee and Peart, 1995). APEs therefore occur as a complex mixture in final effluents of municipal or industrial treatment systems (Lee et al., 1999; Bennie, 1999). The range of concentrations and the relative proportions are dependent on the sources as well as degree and type of treatment (Maguire, 1999; Ahel et al., 1994). As the EO chain length decreases, a corresponding decrease in water solubility is observed. The APs and low chain length APEOs are therefore generally associated with organic particles and sludges in the treatment system. In contrast, NPECs are considerably more water soluble than the corresponding NPEOs and are present in the aqueous phase of final effluent (Maguire, 1999; Bennie, 1999; Servos et al., 2000).

6.3.1.3.2 Environmental concentrations. The environmental concentrations of APEs are generally low except in the vicinity of effluent discharges. However, APEs are commonly found at trace levels in surface waters and sediments around the globe. The relative distribution in water will differ from that in sediments or sludges applied to land and is dependent on the sources, treatment, and environmental characteristics. The differences in the properties of the degradation products make estimation of their environmental

exposure complex, although in general, the AP polyethoxycarboxylates are found in the aqueous phase whereas the short chain APEOs and APs are associated with particles and sludge. Concentrations of APEs near industrial sites or textile industries have been reported to be relative high, for example, >1–1,000 µg/liter (Blackburn and Waldock, 1995; Bennie, 1999; Lye et al., 1999; Ahel et al., 1993). Many of these sites have recently implemented control measures or turned to alternatives, and the environmental release has been reduced (Bennie, 1999; Lee and Peart, 1995; Naylor et al., 1992). A limited amount of data does suggest a decrease in the concentrations of these chemicals in the environment over time (UBA, 2001a). However, APEs continue to be released from a variety of sources, particularly municipal effluent treatment systems. Municipal treatment systems sampled across Canada (Annex I, Table 18) contained concentrations of NP that ranged from <0.02 to 62.1 µg/liter, from 0.12 to 4.79 µg/liter and from <0.02 to 3.20 µg/liter for primary, secondary and tertiary treatment systems, respectively (Bennie et al., 1998; Lee et al., 1998; Servos et al., 2000). In Canadian fresh water, concentrations of NP ranged from non-detectable (<0.02 µg/liter to 4.25 µg/liter (mean, 0.20 µg/liter), with the highest concentrations being in direct proximity to municipal or industrial discharges (Bennie et al., 1997; Bennie et al., 1998). This is consistent with similar surveys of surface waters in the USA (Naylor et al., 1992; Weeks et al., 1996) and Europe (Ahel et al., 1994; Lye et al., 1999; Blackburn et al., 1995; Larsson et al., 1999).

Shang et al. (1999) observed the distribution of NPEs in marine sediments from the Strait of Georgia to be dominated by NP and NP1EO, which were also persistent in sediments. NP concentrations in sediments from the Great Lakes basin and the upper St. Lawrence River ranged from below detection levels (<0.02 µg/g) to 72.2 µg/g d.w. (Lee and Peart, 1995; Bennie et al., 1997; Bennett and Metcalfe, 1998; Bennie et al., 1998). Sediments in rivers from the United Kingdom were reported to have similar levels, from 0.03 to as high as 131 µg/g d.w. in relative contaminated systems (Lye et al., 1997). Sediments concentrations in the River Glatt, Switzerland, were also reported in a similar range (Ahel et al., 1993, 1994). The most common APE detected in sludges is NP, which ranged from 0.74 to 1,260 µg/g d.w., in surveys Canadian treatment systems (Lee and Peart, 1995; Lee et al., 1997, 1998; Bennie et al., 1998; Bennie et al., 1998; Servos et al., 2000). Sludges are routinely applied to amend agricultural soils. There are few data available about the residuals of APEs in these soils, although they do appear to degrade rapidly (Bennie, 1999; Marcomini et al., 1989).

There are minimal data available on APEs in biota in the published literature. Most data suggest a low to moderate potential for these chemicals to bioaccumulate (BAF, 0.9–3,400), but trace levels have been detected in various locations around the globe (Servos, 1999). Concentrations of NP, NP1EO, and NP2EO were as high as 1.6, 7.0, and 3.0 mg/kg, respectively, in four composite samples of fish (chub, barbell, rainbow trout) collected from the Glatt River in Switzerland; concentrations in a single sample of wild duck (*Anas boscas*) ranged from not detected to 1.2, 2.1, and 0.35 mg/kg d.w. for NP, NP1EO, and NP2EO, respectively (Ahel et al., 1993). Eklund et al. (1990) demonstrated some limited bioaccumulation of APEs in marine organisms. Granmo et al. (1991) caged mussels (*Mytilus edulis*) near an outfall of an industrial facility producing surfactants and reported BAFs of 340 (wet weight) for NP. Wahlberg et al. (1990) caged mussels in the effluent of a plant that produced NPEs and measured BAFs of 60 for NP3EO, 100 for NP2EO, 170 for NP1EO, and 340 for NP.

6.3.1.3.3 Effects. Several detailed reviews of the toxicity and bioavailability of APEs have been recently published by Talmage (1994), Staples et al. (1998), Nimrod and Benson (1996), and Servos (1999). Although the data in the literature are scattered among many species, different test methods and chemicals, there is a consistent pattern in the toxicity. NP is relatively toxic (LC_{50}, EC_{50}) to fish (17–3,000 µg/liter), invertebrates (20–3,000 µg/liter) and algae (27–2,500 µg/liter), and chronic toxicity values as low as 6 µg/liter in fish and 3.7 µg/liter in invertebrates have been reported. Although there are considerably fewer data available for the NPEOs and NPECs, there is an apparent increase in the toxicity of NPs and NPEs with decreasing EO chain length. NPECs that are much more water soluble are much less toxic than are corresponding NPEOs and have acute toxicity similar to NPEOs with 6–9 EO units. The potential of these compounds to bioaccumulate is dependent of their hydophobicity and varies greatly, with APs having only a moderate tendency to accumulate in biological tissues.

Numerous studies have demonstrated the ability of NPEOs and their degradation products to disrupt the normal function of the endocrine systems of various organisms. Soto et al. (1991) accidentally found that *p*-NP released from polystyrene centrifuge tubes caused proliferation of human estrogen-sensitive MCF-7 breast tumor cells (E-screen). They have since been reported to cause a number of estrogenic responses in a variety of aquatic organisms, including altered growth of testes (Ashfield et al., 1998), altered steroid metabolism (Tremblay et al., 1998), intersex (Gray and Metcalfe, 1997), histological changes (Miles-Richardson et al., 1999), and disruption of smoltificaton (Madsen et al., 1997; Fairchild et al., 1999).

APEs, especially NP and octylphenol, bind to the ER, resulting in the expression of several responses both *in vitro* and *in vivo*, including the induction of vitellogenin in fish (Jobling et al., 1996). These effects occur at a range of concentrations similar to those at which chronic effects occur in fish and invertebrates. The threshold of NP for induction of vitellogenin in rainbow trout was reported as 10 µg/liter (Jobling et al., 1996), whereas the induction of mRNA for vitellogenin was reported at concentrations as low as 1 µg/liter (Fent et al., 1999). Recent reports by Miles-Richardson et al. (1999) suggest that in fathead minnows, histological and biochemical effects can occur at concentrations approaching or below 1 µg/liter. However, the significance of these responses is not fully understood, and the effects on the organism or population have not been determined. It has been demonstrated that NP can affect smoltification, resulting in reduced growth and survival in Atlantic salmon (*Salmo salar*) after very short-term exposures to concentrations expected in the environment (Madsen et al., 1997). Intersex in Japanese medaka has been demonstrated at 50 µg/liter (Gray and Metcalfe, 1997). Although potential effects mediated through the ER have been identified both *in vitro* and *in vivo* for NP in fish, this is only one mechanism by which a chemical such as NP can potentially interact with endocrine systems. As with acute and chronic toxicity, there are few data available on the relative potency/estrogenicity of the other metabolites, and there is considerable discrepancy among the few existing studies. Jobling and Sumpter (1993) reported that using trout hepatocytes to measure induction of vitellogenin, NP2EO, and NP1EC were 0.67 and 0.63, the potency of NP.

The importance of considering APEs as a mixture rather than individual compounds has been demonstrated by Servos et al. (2001). In municipal effluents, other compounds such as natural

and synthetic estrogens have been identified that also bind to the ER and cause similar biological responses as APEs (Desbrow et al., 1998; Routledge et al., 1998). In some effluents, APEs may contribute only a small proportion of the total estrogenic potency, although this will vary drastically depending on the sources, treatment, and compartment assayed.

6.3.1.4 Summary and conclusions of wildlife exposures. The case studies summarized in this section, along with the data presented in Annex I, clearly provide evidence that considerable exposure to potential EDC s (particularly POPs) has occurred in a variety of wildlife species. However, most of these exposure data come from selected species at the top of the food chain or from wildlife living in a contaminated habitat in Europe and North America. Exposure data for nonpersistent EDCs, for other wildlife species, at low environmental levels, and in other parts of the world are generally lacking. Even with the availability of good data sets, there are difficulties in comparing exposure levels between species, over time, and in different areas because of different approaches to sampling, analytical methodologies, data reporting, and statistical treatment. To adequately assess EDC-related effects in wildlife, global, long-term exposure monitoring is needed, using harmonized, consistent methodologies to ensure comparability of data. Obviously, it is impossible to measure all areas and all species at all life stages; therefore, a strategic approach needs to be developed to determine what types of exposure data are needed to adequately assess the impact of EDCs on wildlife.

6.3.2 Human Exposures—Some Selected Case Studies

Some case studies are summarized to illustrate human exposure to a diversity of potential EDCs. These include dioxinlike compounds (such as PCBs, OH-PCBs, PBDEs, DDTs), phthalates, atrazines, and phytoestrogens. Some information on concentrations of potential EDCs in breast milk and potential exposures of children and vulnerable populations to EDCs is also summarized.

6.3.2.1 "Dioxins." PCDDs and PCDFs, known collectively as dioxinlike compounds, are by-products of a variety of industrial and thermal processes. There are 75 and 135 congeners of PCDDs and PCDFs, respectively; however, only 7 PCDDs and 10 PCDFs are generally found in humans. Unfortunately, these include the most toxic 2,3,7,8-substituted congeners. Two chemical subclasses of PCBs, the non-*ortho*-chlorinated PCBs and the mono-*ortho*-chlorinated PCBs, have toxicological properties similar to the PCDDs and PCDFs and are also discussed here. The ingestion of food containing trace levels of these chemicals is estimated to account for more than 90% of human background body burden.

PCDD and PCDF levels are normally reported in various matrices as concentrations of individual PCDD/PCDF congeners or as total dioxin concentration per gram of lipid in the sample calculations. Total concentrations of "dioxinlike" compounds are often calculated and reported as TEQs, which are sums of concentrations weighted to account for the varying potencies of the different compounds. WHO has established a tolerable daily intake for the whole group of dioxinlike compounds of 1–4 pg TEQs/kg body weight/day (Van Leeuwen and Younes, 2000).

6.3.2.1.1 Trends. It is clear from many reports that there has been a marked decrease over the past 20–30 years in the levels of PCDDs/PCDFs in the general population of industrialized countries (Furst et al., 1992; Päpke, 1998; Liem et al., 2000; Meironyté and Norén, 2001). Some recent data indicate that this trend may not be continuing in Germany and Spain (Liem et al., 2000; Fürst, 2001).

6.3.2.1.2 Levels. Several review articles have described levels of PCDDs, PCDFs, and the dioxinlike PCBs in the general population. Data are summarized in Annex I (Tables 19 and 20). Data sets are from New Zealand (Bates et al., 1999), the USA (Anderson et al., 1998), Norway (Johansen et al., 1996), Sweden (Norén and Meironyté, 2000), and Canada (Dewailly et al., 1996). Samples collected during the 1990s from different matrices (blood, serum, breast milk) all indicate TCDD levels in the range of 2–3 pg/g lipid weight (ppt). This level is consistent with the reported level of 3.4 ppt in the fat of breast milk from 33 countries (IARC, 1997a). For some PCDDs, the reported values from Europe and New Zealand tended to be lower than those from North America (Wingfors et al., 2000). In contrast, 2,3,4,7,8-PCDF levels occur in higher concentrations in samples from Europeans compared with those from North Americans and New Zealanders. The higher concentrations of 2,3,4,7,8-PCDF in the European samples largely accounts for the higher contribution to the TEQ. The TEQs of the PCDDs and PCDFs in these five data sets range from 12.6 to 24.2 ppt, which is in agreement with the range of 4–27 ppt TEQ in breast milk samples taken in 1993–1994 in 18 countries (see Figure 6.5). Although levels of TEQs in industrialized countries are very consistent (Schröter-Kermani et al., 2000; Päpke et al., 2000), there may be regions where higher levels ("hot spots") occur. For example, higher TCDD levels have been reported in serum and breast milk from residents of Kazakhstan, with levels in the range of 6.9–68.6 ppt TEQ and 1–208 ppt TEQ, respectively (Hooper et al., 1998, 1999). Unfortunately, there are many parts of the world where biomonitoring has not been carried out.

The temporal concentration differences noted above for the PCDDs and PCDFs with the time of sample collection are not seen for the non-*ortho*-substituted PCBs. The earliest samples from the USA have the lowest levels of these PCB congeners. The TEQs for the non-*ortho*-substituted PCBs from the two Scandinavian data sets are four or five times higher than the U.S. samples. The reasons for this variance are not known and more complete data sets are needed to better describe total TEQ levels. Mono-*ortho*-substituted PCBs (e.g., PCBs 105, 118, and 156) can contribute greatly to the TEQ and should be included in calculations of TEQs.

Several investigators have noted increased levels of the above chemicals in humans with increasing age (Bates et al., 1999). Bates et al. (1999) observed a three- to fourfold increase in serum of New Zealand individuals 65 years of age and older compared with the 15–24-year age group. Levels for males and females were similar. In contrast, plasma levels of TCDD in women in Seveso, Italy, had higher levels than men, and this gender difference persisted after adjustment for location within the zone, age, body mass index, and smoking (Landi et al., 1998; Päpke, 1998).

6.3.2.2 PCBs. The manufacturing of PCBs has been banned in many countries since the 1970s (de March et al., 1998). However, because of their long lifetime in old electrical equipment, continued use in some parts of the world, and their persistence and bioaccumulation, populations continue to be exposed to PCBs. For toxicological purposes, the PCBs have been divided into three main classes: those containing no *ortho* substitution (non-*ortho*-PCBs), those with one chlorine in the *ortho* position (mono-*ortho*-PCBs), and those containing chlorine substitution at two or more *ortho* positions. The first group acts through mechanisms similar to dioxins and may be analytically measured in the same fraction of the

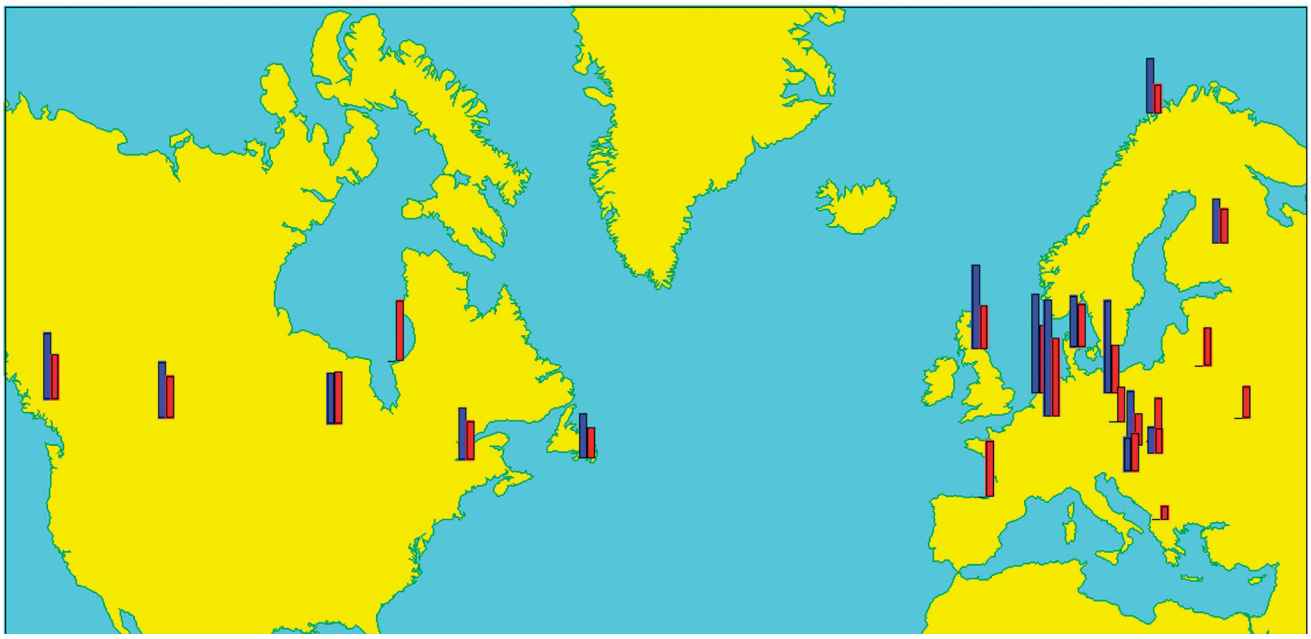


Figure 6.5 - TEQs (pg/g lipid weight) and PCB concentrations (ng/g lipid weight) in human milk from 18 countries around the world. TEQs 1987/88 = blue bars and 1993/94 = red bars; highest bar = 40 pg/g TEQ lipid weight.

sample preparation; hence, they are generally reported on a lipid-adjusted basis. The mono-*ortho*-PCBs are generally not analyzed in the same fraction as the PCDDs and PCDFs but have been assigned TEFs (Ahlborg et al., 1994). However, this group is often reported on a whole weight (or volume) basis rather than a lipid-adjusted basis and thus cannot be accurately included in the total TEQ. The group containing chlorine substitution at two or more *ortho* positions is often the highest in concentrations in environmental and biological samples. Historically, they were often reported only on a whole-weight (or volume) basis (ng/g or ng/ml) in biological samples. The three congeners generally found in the highest concentrations in biological samples are: 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB 153); 2,2′,3,4,4′,5′-hexachlorobiphenyl (CB 138); and 2,2′,3,4,4′,5,5′-heptachlorobiphenyl (CB 180). Prior to the 1990s, most of the PCB exposure data in humans were reported as total PCBs. Since that time, exposure to PCBs has generally been reported on an individual PCB congener basis. The different ways of reporting PCB concentrations lead to major difficulties in making data comparisons. To promote future comparisons of data, measurement of congener specific concentrations should be encouraged.

6.3.2.2.1 Levels in special populations—fish eaters. Svensson et al. (1995) showed a high correlation between consumption of fish from the Baltic Sea and PCDD, PCDF, non-*ortho*-PCB and mono-*ortho*-PCB levels. Three groups were studied: individuals who did not consume any fish, moderate fish consumers (200–500 g of fish/week), and high fish consumers (consumption rate of 700–1,750 g of fish/week). The average total TEQ in serum for these three groups was 17.5 ppt, 25.8 ppt, and 63.5 ppt, respectively. The non-*ortho*-PCB contribution to the total TEQ in the nonfish/fish consumers from Sweden was 30%. Other studies on PCB levels in populations with high seafood intake from Latvia and Sweden (Sjödin et al., 2000); The Netherlands (Hanrahan et al., 1999) and the Great Lakes area (Anderson et al., 1998) have reported higher total PCB levels in high-fish-consuming populations compared with the concentrations in populations eating little or no fish. Recently, high PCB concentrations (CB 153) were reported in a subgroup of pregnant women from the Faroe Islands (Fängström et al., 2002). The AMAP, analyzed PCB levels in maternal plasma in 1995/1996 from six of the eight circumpolar countries, and the results are summarized in Annex I, Table 20. Levels have also been measured in breast milk. These results are summarized in Annex I, Table 21. In general, PCB concentrations and concentration patterns were similar in all countries except Greenland, where higher concentrations were found. One group of males (consumers of Norwegian crabs) also had much higher median levels of PCBs than did other AMAP participants (Jacobson et al., 1996). Studies are difficult to compare because of different sampling, analytical and reporting methods, but overall the body of evidence indicates that these populations have increased exposures.

6.3.2.2.2 OH-PCBs. PCBs are transformed to OH-PCBs in both wildlife (Jansson et al., 1975) and experimental animals (Sundström et al., 1976). Several reviews on the metabolism of PCBs to OH-PCBs have been published (Safe, 2000; Letcher et al., 2000). In general, OH-PCBs are excreted, but some may also be retained in blood (Bergman et al., 1994, Fängström et al., 2002). More than 40 OH-PCBs are present in human blood, of which 39 have been identified (Hovander et al., 2001). The OH-PCBs are present in blood at slightly lower concentrations than are the parent PCB congeners (Sandau et al., 2000; Sjödin et al., 2000) but at much higher concentrations in blood than in adipose tissues (Bergman et al., 1994; Meironyté Guvenius et al., 2002). The lipid content of the blood compartment does not affect the retention of OH-PCBs, because they are bound to plasma proteins (Brouwer et al., 1998). The major OH-PCBs in human blood are metabolites of the highly chlorinated PCBs with the hydroxy group preferentially in the 4-position but occasionally in the 3-position (Bergman et al., 1994; Sandau et al., 2000; Sjödin et al., 2000; Hovander et al., 2001). Levels of OH-PCBs in human blood samples from northern Canada, Latvia, Sweden, and the Faroe Islands range between 10% and 20% of the parent PCB concentrations.

6.3.2.3 PBDEs. As noted above, the plasma concentrations of PBDEs in humans from industrial countries do not appear to be decreasing. PBDEs are potential EDCs because both PBDE and their metabolites (OH-PBDEs) may interfere with the estrogen and/or thyroid system (Meerts et al., 2001). PBDEs are used as flame retardants in high-impact polystyrene, flexible polyurethane foam, textile coatings, wire and cable insulation, and electrical connectors. It has been reported that PBDEs may be released from television sets and computers and then absorbed into the lipid portions of the body (Sjödin et al., 1999, 2000). Even though data are limited, it is clear that PBDEs are bioavailable and accumulate in human tissues. The half-lives of polybrominated diphenyl congeners vary with the number of bromine atoms (Sjöden, 2000). The major PBDE congener present in human blood or breast milk is 2,2′,4,4′-tetrabromodiphenyl ether (Meironyté, 1999; Ryan and Patry, 2000; Strandman, 2000; Schröter-Kermani, 2000; Sjödin et al., 2001). Other PBDE congeners that have been detected, include the high-molecular-weight compound decabromodiphenyl ether (Sjödin et al., 1999, 2001). Higher levels of these PBDEs have been measured in personnel dismantling electronic products and in computer operators compared with controls (Jakobsson et al., 2002). Human exposure data, as determined in Canada, Germany, Latvia, Sweden, and the USA, are summarized in Annex I, Table 23. There is some evidence that the levels in general are higher in North America than in other parts of the world (She et al., 2000; Päpke et al., 2001; Ryan and Patry, 2001), but further monitoring data are needed.

6.3.2.3.1 Temporal trends. In contrast to the chlorinated POPs, the concentration of the PBDEs in human breast milk samples from Sweden has doubled every 5 years from 1972 to 1997, as shown in Annex I, Figure 21 (Norén and Meironyté, 2000). Some preliminary time trend data from breast milk in Canada (Ryan and Patry, 2000) and from blood in Germany (Schröter-Kermani et al., 2000) also indicate increased levels of PBDEs over time. In the last couple of years, however, the levels of one of the major PBDE constituents, BDE-47, have started to decrease in mother's milk (Meironyté Guvenius, 2002). Humans are exposed to PBDEs via contaminated food, such as fatty fish and whale blubber (de Boer et al., 2000), and via inhalation (Sjödin et al., 2000).

PBDEs are metabolized to hydroxylated compounds and possibly sulfur-containing metabolites (Hakk et al., 1999; Örn et al., 1998). A few OH-PBDEs have been reported as naturally occurring chemicals produced in marine algae and sponges (Gribble, 2000). Blood levels of OH-PBDEs are similar to those of the PBDEs (Asplund et al., 1999, 2001; Hovander et al., 2001).

6.3.2.4 DDT. The manufacturing and use of a number of OC pesticides, including DDT, have been banned or greatly restricted in many parts of the world since the 1970s. DDT, however, is still used in some developing countries, primarily for public health control of vector-borne diseases in the southern hemisphere. While the concentrations of DDT and the DDT metabolites DDE and methylsulfonyl-DDE are decreasing in the northern hemisphere, these compounds are still present at relatively high concentrations in some populations. Norén and Meironyte (2000) reported that levels of 4,4′-DDT and its metabolite 4,4′-DDE in Swedish human milk samples decreased dramatically during 1967–1997. The levels of dieldrin also decreased at the same rate. In contrast, measurements of DDT and its metabolites in 50 human milk samples collected in Mexico City in 1995 still showed relatively high levels (Torres-Arreola et al., 1999). Concentrations up to 50,000 ng/g lipid weight and more of DDT was also seen in blood samples (Annex I). Figure 6.6 summarizes concentration ranges of DDT, lindane, and HCB in different parts of the world. These data indicate that levels are higher in geographical locations where certain chlorinated insecticides are still used, but in general, low levels of these persistent compounds are found globally.

6.3.2.5 Phthalates. Phthalates are diester derivatives of phthalic acid used primarily as plasticizers to make plastic products more flexible. Tetrabromophthalate diethylhexyl ester is used as a flame retardant. Certain plastics may contain up to 40% phthalate by weight. Consumer products containing plastics include imitation leather, rainwear, footwear, upholstery, flooring, tablecloths, shower curtains, food packaging materials, children's toys, tubing, and containers for blood transfusions and blood products. Because these plasticizers do not become a permanent (chemically bonded) part of the plastic matrix during the manufacturing process, they can migrate from the plastic product to environmental matrices under certain conditions. As a result, they have become ubiquitous in our environment, and people may be continually exposed to low levels of phthalate esters. Of particular recent concern is the potential for phthalate ester exposure to young children orally by chewing on toys, teething rings, and pacifiers containing these plasticizers (Steiner et al., 1999). Once in the body, phthalates are quickly metabolized to the corresponding monoester metabolite, which is then rapidly eliminated in the urine as its glucuronide conjugate or further metabolized. Concern has been raised about phthalate esters in relation to reproductive effects on adult males and development of male offspring (see Chapters 3 and 5).

Few assessments of human exposure to phthalates have been reported. The major phthalates used in commerce are the diethyl, dibutyl, dicyclohexyl, butyl, benzyl, di-2-ethylhexyl, di-*n*-octyl, di-iso-nonyl, and di-isodecyl. Exposure to phthalates was measured (Blount et al., 2000a, 2000b) in almost 300 urine specimens collected from adults in the U.S. NHANES III (1988–1994) survey. The phthalate monoesters with the highest urinary levels were monoethyl phthalate (95th percentile, 3750 ppb; median, 305 ppb), monobutyl phthalate (95th percentile, 294 ppb; median, 41.0 ppb), and monobenzyl phthalate (95th percentile, 137 ppb; median, 21.2 ppb). reflecting exposures to the parent compounds diethyl phthalate, dibutyl phthalate, and benzyl butyl phthalate. The authors speculated that metabolites of the more lipophilic phthalates, such as diethylhexyl phthalate, may be excreted via the bile and into the feces.

6.3.2.6 Atrazine. Atrazine is a member of the triazine herbicide family and has been widely used for weed control in agricultural crops. It is frequently found in surface water and groundwater. Because it may be found in drinking water, its use has been banned or severely restricted in many countries. In the context of EDCs, there is concern about atrazine related to the development of mammary tumors in exposed rats (see Chapter 3). Human exposure to atrazine has been assessed primarily by measurement of its metabolites in urine (Barr et al., 1999; Catenacci et al., 1993; Lucas et al., 1993) or less frequently in blood. Limited data of internal atrazine exposure levels in human serum indicated concentrations in the ppt range and in urine in the ppb range (Barr et al., 1999; Beeson et al., 1999).

6.3.2.7 Phytoestrogens. Seven phytoestrogens or their metabolites have been measured in 200 human serum and urine specimens from adults participating in the NHANES III survey, but the results are not yet available. Preliminary data indicate that the levels were higher in the urine compared with the serum

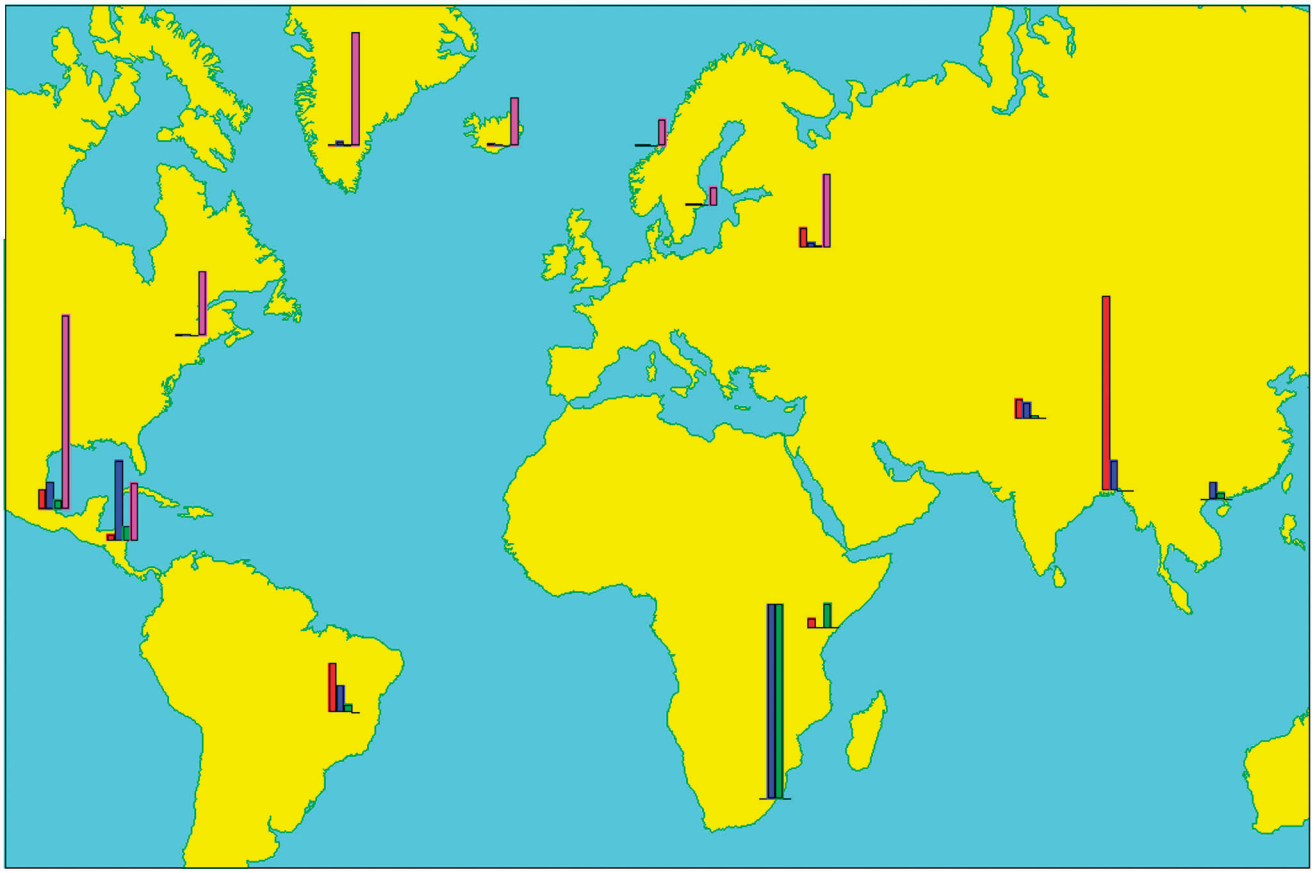


Figure 6.6 - DDT and other pesticides in humans around the world. Red bars = β-HCH (560 ng/g l.w. in Brazil), blue bars = DDE (2.200 ng/g l.w. in Mexico), green bars = DDT (780 ng/g l.w. in Kenya), purple bars = HCB (980 ng/g l.w. in Greenland).

samples. The urine results did not differ greatly from the mean of the urinary levels reported in the literature from Western populations known to consume phytoestrogen supplements. Horn-Ross et al. (1997) examined urinary levels of several phytoestrogens in samples from a multiethnic population of young women in the San Francisco Bay area. The highest urinary levels of coumestrol, lignans, enterodiol, and enterolactone were found in white women, and the lowest levels in Latin and African-American women. Isoflavone levels were generally similar in all groups, but higher genistein levels were observed in Latin women.

Some mycotoxins (low-molecular-weight, cyclic metabolites produced by different species of fungi) have been shown to possess estrogenic potential. The primary route of general human exposure to these mycotoxins is via the food chain, but occupational exposures (e.g., processing peanuts and corn) may occur via inhalation. There is a paucity of data on human exposures to mycotoxins. The daily exposure of young Canadian children to zearalenone from food consumption has been estimated to be in the range of 0.05–0.10 µg/kg body weight/day (Kuiper-Goodman et al., 1987).

6.3.2.8 Conclusions on human exposures. There is still considerable uncertainty on associations between human health effects and exposure to EDCs. To resolve uncertainty, better exposure data must be generated. This is true for the general population as well as for the more susceptible subpopulations. Environmental human exposure to most EDCs occurs primarily via ingestion of food. Inhalation and dermal routes of exposure are generally not important. To date, exposure levels have primarily been measured in adults, and data on exposure(s) during critical development stages (e.g., fetus, infants and children) are urgently needed. Estimates of fetal exposures are generally calculated from maternal levels, but these measurements may not accurately reflect exposure during critical stages of fetal development. Fetal samples such as cord blood and amniotic fluid may also not reflect exposure during critical developmental periods. Meconium may be a more representative biological sample to assess fetal exposure. Additional data are needed on the distribution of EDCs within the body, on correlations among tissue levels, and on excretory products. Computer-generated exposure models need to be validated. Many EDCs occur in mixtures of related chemicals, posing particular measurement problems, and/or as part of complex mixtures. As yet, we know little about the relative importance in terms of hormonal activity of related chemicals and perhaps even less about complex mixtures. Mechanisms need to be developed to prioritize globally the most important potential EDCs. Mechanisms are also needed to promote better exchange of information with the intention of improving the comparability of national and regional monitoring programs for EDCs.

6.4 Measurement of Exposure to EDCs

Measurements of EDC residues may utilize special instruments (gas–liquid or high-performance liquid chromatography, mass spectrometry) similar to those used for other environmental contaminants. However, methods that rely on biological activity, including some ELISAs and assays that depend on protein-receptor binding, are finding increased utility particularly as screening

tools, because the chemical nature of an endocrine-disrupting sample may not be known, and biological activity may be the best (or only) indicator of the presence of EDCs. A tiered approach utilizing a combination of measurement methods is often desirable. In order to ensure high-quality data, QA procedures should be applied to all steps involved in sampling, analysis, data processing, and compilation of results (IPCS, 1992).

6.4.1 Sampling

Key issues in collecting samples to measure EDC exposures include the following:

1) *representativeness of the samples*: exposure may be assessed for a number of different reasons that may influence the choice of sampling locations or sampling matrices;
2) *timing and frequency of sampling*: collection of samples should address exposure of greatest concern (e.g., long-term chronic exposure vs. intermittent short-term exposure), patterns of contamination (e.g., continuous discharge vs. one-time accidental contamination), and the end points of greatest concern;
3) *selection of matrices*: the choice of matrix is determined by factors such as relevance to route of exposure, ease and practicability of sampling, which analytes are to be assessed, and in some cases, ethical considerations;
4) *statistics*: appropriate statistical methods need to ensure the relevance of sampling (e.g., pooled vs. individual samples, numbers of samples from different locations/species); and
5) *sample storage and preservation methods*: special methods may need to be used to avoid contamination with other chemicals with potential EDC activity that are commonly found in sampling and laboratory equipment (e.g., plasticizers).

6.4.2 Analytical Considerations

6.4.2.1 Measuring specific chemicals. Established analytical methods are readily available for most EDCs. Many countries have established regulatory authorities or requirements to provide standards for chemical analyses and methods for testing for residues in food or the environment. In developing countries, approaches for monitoring pesticide exposure are generally poorly developed and vary dramatically (Gonzales, 1999). A number of international organizations, including the International Organisation for Standardization, the Association of Official Analytical Chemists International, International Union of Pure and Applied Chemistry, CODEX, and the Organisation for Economic Co-operation and Development, have initiatives to standardize methods and promote established protocols for producing acceptable data (Ambrus, 1999).

Most of the environmental monitoring studies of OCs have an unintentional bias because of the use of an electron capture detector that selectively detects halogen-containing chemicals from nonhalogenated contaminants. Because these OCs also tend to be more persistent and bioaccumulate, scientists have focused disproportionately on these chemicals. A large array of other potential EDCs (e.g., APs, bisphenol A, 2,4,6-tribromophenol, tetrabromobisphenol A, and the OH-PCBs) may be overlooked because of lack of comparable analytical specificity. A number of phenolics are also naturally produced (Gribble, 2000), and these chemicals have received little attention in terms of broad EDC monitoring programs.

Multi-residue methods have the ability to test for a broad range of chemicals in a single sample and therefore are useful for screening purposes. There are multi-residue methods tailored for OCs, organophosphates, and dibenzodioxins/dibenzofurans. Other classes of chemicals (e.g., phenolics, steroids, carbamates) are analyzed by class-specific or chemical-specific methods. The application of directed analysis permits more specificity and is more likely to identify previously overlooked contaminants. Multicompound methods used in food testing could be adapted for use with other biological matrices (Seiber, 1999).

6.4.2.2 Identifying unknown samples. Biological methods can be used as general screens to determine whether EDC-active chemicals are present or not in a given environmental sample, but they are limited in their ability to identify specific chemicals. In order to verify the identity of the causative agent and to quantify the EDCs present, classical chemical methods should accompany biological techniques (Cech et al., 1998). Chemical analyses of EDCs are similar to those employed for other organic residues. The preferred methods for EDCs are those that provide a maximum of structural information about the chemical at low concentrations (e.g., mass spectrometry) combined with biologically based analytical methods (see below).

6.4.2.2.1 Biologically based methods. The major biological methods currently available for detecting hormonally active substances are *in vitro* bioassays for assessing estrogenic or anti-estrogenic substances. Methods are also being developed to detect androgens and antiandrogens, thyroid-active chemicals, and chemicals that interfere with steroid biosynthesis and metabolism. International and national efforts are ongoing to develop standardized and validated test guidelines to identify, screen, and detect EDCs (OECD, 1999b). *In vitro* bioassays have several disadvantages, including the inability to account adequately for *in situ* bioaccumulation, lack of metabolic capacity, and the fact that they are generally specific for only one mechanism of action (e.g., receptor binding). A battery of screens must be employed if all possible mechanisms of endocrine disruption are to be addressed (Matthews et al., 2000).

Biologically based methods can be categorized as follows:

a) *Receptor binding assays* measure binding of agonists or antagonists to a specific cellular receptor. These methods rely on isolation of receptor ligands from test organisms (often mice or rats), which are then co-incubated with a high-affinity radioligand and with various concentrations of the test compound or mixture. Displacement of the radioligand is monitored against a known active compound, often E_2. It does not take into account pharmacokinetics and metabolic effects, which would be part of an *in vivo* interaction (Kramer et al., 1997). The concentration range for effects for some of those tests can be extremely low (i.e., 0.06–0.2 ppt), which is within the range of detectability of many modern analytical methods (Wooge et al., 1992; Soto et al., 1995).

b) *Cell proliferation assays* depend on the ability of estrogens to induce cellular proliferation in target organs such as rat pituitary cells and several human breast cancer cell lines (e.g., MCF-7 and T47-D cells). Cell proliferation is considered a hallmark of estrogen action (Hertz, 1985) and can be induced with very low levels of estrogenic substances (Soto et al., 1997).

c) *Receptor-dependent gene expression assays* measure the ability of a compound to stimulate a receptor-dependent response in genes or induction of gene expression proteins in isolated cell lines. Various assays rely on use of transfected mammalian or yeast cells that trigger detection of a linked gene expression (e.g., production of an enzyme to degrade a sugar); the concentration of sugar remaining after the test is related to the potency of the chemical. These assays also do not address pharmacokinetics of the chemical. An *in vivo* assay has been developed in Zebra fish where a receptor-dependent gene is stably introduced into the whole organism (Legler, 2000). In this assay, a luciferase gene induction is utilized

to detect transactivation of a transgenic ER in whole-organism exposures to zebrafish with measurable responses down to 0.1 nanomolar E_2.

d) *Immunoassays* can detect the presence of a specific chemical at low, biologically relevant levels (Moye, 1999a). There are only a few classical chemical detection methods that are as effective as the ELISA methods in detecting chemicals below the parts per billion range. For classical methods to achieve comparable detection limits, extra efforts are needed to enhance the analyte's detectability through preconcentration, derivatization, or reliance on ultrasensitive detection methods (e.g., electron capture) (Moye, 1999b). On the other hand, immunoassays may lack the specificity of chemical detection methods.

6.4.2.2.2 TIE approach. The TIE approach utilizes *in vitro* bioassays in conjunction with chemical methods for identifying EDCs. The technique involves the use of toxicity-based fractionation procedures that can be utilized to identify endocrine-active constituents in media such as water and food (Mount et al., 1988). The challenge is to combine an effective method for fractionating and isolating the compounds of interest with a bioassay that distinguishes end points relevant to the effect under examination. Bioassays that have been used in TIE studies include the ER CALUX for planar PCBs, dioxins, and furans (Pauwels et al., 2000); the ER CALUX for ER agonists; recombinant yeast assays (Routledge et al., 1996); and reporter gene assays (Snyder et al., 2000). A recombinant yeast TIE system has been used to assess sewage treatment effluents (Desbrow et al., 1998), and a reporter gene assay has been used for water samples (Snyder et al., 2000). TIE methods are useful where some components of the mixture are clearly more hormonally active than others. TIE methods have been proposed for use on plasma to assess the relative contributions of endogenous versus exogenous hormones (Sonnenschein et al., 1995; Soto et al., 1997).

6.4.3 Mixtures

Many potential endocrine disruptors exist as mixtures of related isomers and congeners) (see Table 6.2). Individual chemicals within these mixtures may vary greatly in potency and may interact with each other in an unpredictable manner.

Selective identification methods are available for related isomers, congeners, and homologues, but these require much time and effort. Analytical methodr for such mixtures ultimately require specific isomer standards for the correct quantification and identification. These are available for some (e.g., PCBs, PCDDs, PBDEs) but not for all classes of EDCs. Although historical data generated for mixtures continues to be of value, efforts need to be made to relate this information to data now available on the individual components, so that previous exposures can be related to potential long-term effects. There is also need to continue to monitor for mixtures utilizing the best available technology so that individual isomer data as they becomes available can assist in reconstructing exposures.

6.4.3.1 Chirality considerations. The concept of chirality relates to the spatial, three-dimensional configuration of organic chemicals or the existence of nonsuperimposable mirror images (Kallenborn and Hühnerfuss, 2001). For chemicals that have asymmetric centers or isomers formed by hindered structural types (atropisomers), two different chiral forms exist for each center/type. As the number of centers increases, there is a geometric progression of possible configurational forms. Each possible configuration may have very specific biological effects that must be considered in any assessment of cause and effect. Some EDC effects are dependent on receptor binding, which depends on configurational parameters, and characterization of these chiral forms is critical.

Many PCB congeners exist as chiral pairs or atropisomers (Rodman et al., 1991; Wong et al., 2000), as do PCB methyl sulfones (Ellerichmann et al., 1998) and many of the individual toxaphene isomers and chlordane components (Kallenborn and Hühnerfuss, 2001). NP mixtures may contain as many as 120 separate structures, with several likely to have chiral forms. Chiral selectivity for the endocrine effects of *o,p′*-DDT has recently been reported. (Wiese et al., 1999) found that the *R*(–) enantiomeric form of *o,p′*-DDT displayed enhanced binding activity for ER-α. *R*(–) *o,p′*-DDT was also found to be significantly more potent than the *S*(+) form in estrogen-dependent reporter assays (Wiese et al., 1999). The enantiomer-selective antiandrogen capacity of *o,p′*-methoxychlor was also observed (Wiese et al., 1999). Chromatographic methods have been used to separate chiral forms of a number of pesticides (Buser et al., 2000; Garrison et al., 1996; Müller et al., 1992; Ren et al., 2000).

6.4.3.2 TEF/TEQ approaches. It has been suggested that approaches similar to the TEF/TEQ systems developed for dioxins

Table 6.2 - Common Environmental Mixtures of EDCs

Compound Name	Likely Number of Components	Number of Identified Components	Possible Chiral Forms	Commercial Standards Available	
				Components	Chiral Forms
Tech-DDT	6	6	2	6	2
Tech-chlordane	7 major (>120 minor)	~120 (more when weathered)	4 major (~60 minor)	4	3 (major components)
Toxaphene	>200	13 (97%	3 identified proposed)	9	None
PCBs	209	209	19 (atropisomers)	209	None
NP	40	23	>30	None	None
PCDDs	19	19	0	19	None
PCDFs	16	16	0	16	None
Polybrominated diphenyl ethers	209	~15	0	41	None
Phthalates	18	18	2	7	None
HCHs	4	4	1	4	1
Endosulfan	2	2	2	2	2

and dioxinlike chemicals could be developed for EDCs (see section 6.3.2.1). This approach simplifies the handling of data on these compounds and gives a single measure of the toxicity of a sample. Various systems for weighting the amounts of individual congeners in a mixture have been used (WHO, 1997). A similar approach for EDCs might involve reporting activity in environmental samples in terms of E_2 equivalents. This approach has been used by Servos et al. (2001) to report NP activity in environmental samples in terms of total E_2 equivalents.

6.4.4 QA/QC

The importance of QA/QC procedures in assessing exposure to chemicals has been reviewed (IPCS, 1992). Without adequate QA/QC procedures, it is difficult to compare global monitoring data. Certain features are especially important for measuring EDC compounds; these include matrix spikes, precision measurements, spike recovery studies, detection limit determination, method validation, continuing calibration checks, quantitation with standard curves with known reproducibility, frequent documentation, adherence to standard operating procedures, instrument performance checks, standard expiration date adherence, intralaboratory comparisons when possible, analysis of reference standards, and frequent QA audits (Fong, 1999). The application of QA/QC to biological measurements is equally important. QA/QC procedures must address expected EDC concentration and biologic variation in the study performed (Bignert et al., 1994).

There is a large variation in detection limits for EDCs. Specific OCs have the lowest detection limits because of their responsiveness to electron-capture gas chromatography and gas chromatography/mass spectrometry. Detection limits less than 0.001 ng/liter in water are achievable by high sample volume preconcentration methods. Detection limits for OC mixtures (PCBs, chlordane, toxaphene) are higher than for specific OC compounds. For nonhalogenated compounds (e.g., phthalate esters), detection limits are often 100 times higher than for halogenated EDCs. For the more polar EDCs, such as E_2 and nonyphenols, detection limits are also higher than for organohalogens. These differences in ddtection limits may introduce a bias toward generating more data on persistent EDCs. Newer, highly sensitive methods, which can detect a broader spectrum of EDCs, are being developed and are beginning to have an impact in the field of environmental exposure analysis for EDCs. When relating exposure to effects, detection limits need to be considered.

6.4.5 Exposure Models

An exposure model is an empirical framework, which allows estimation of exposure parameters from available input data. Chemical release estimates, fate and transport modeling, and exposure potentials based on life habits can be employed to estimate exposure for wildlife and/or humans by way of air, food, or water or in total. Models vary in their sophistication, geographic scope, data input needs, and requisite computational power (Calimari, 2001; SETAC, 1994; Mackay, 2001, IPCS, 2000). Bioaccumulation potential can be estimated using existing models and these may provide estimates of external exposure concentrations without conducting lengthy monitoring and assessment programs (Sharp and Mackay, 2000). A widely accepted model for bioaccumulative exposure in humans is described in the US EPA's document on human health risk from dioxins due to combustion facilities (Sharp and Mackay, 2000; US EPA, 1994, 1998a, 1998b).

Calibrated exposure models for nonpersistent EDCs are not common, because there are currently few exposure data available to validate the models. Exposure estimates for these chemicals may need to be derived from surrogates. Under the European Existing Substances Regulation, modeling of potential exposure has been carried out for chemicals such as NP, bisphenol A, and several phthalates. Models for estimating human exposure to pesticides through food consumption are described in many standard toxicology texts and have also been regularly updated for pesticides using market basket data and verification through monitoring programs (NRC, 1993; Olin, 1998; IPCS, 2000). These models would be applicable to nonpesticide EDCs but have not been used to date, probably because there is no regular monitoring system for most of these compounds.

The PCB food-chain model developed by (Thomann et al., 1984) was used to project future residue levels in Hudson River and potential exposure to humans. Thomann et al. (1984) modeled feeding and gill absorption as the primary routes of uptake. For human consumption of foods from animal origin there is a model for TCDD transfers from incinerator emissions to humans (Fries and Paustenbach, 1999). Current models based on adult dosing are inadequate for estimating exposures *in utero* or neonatally. The present models need to be validated by comparison to monitoring data before they can be applied more generally.

6.4.6 SARs

Structure–activity methods can be used to estimate the potential EDC activity of untested chemicals. So far, most progress has been made with structures that bind to the ER. If the bioassay test is based upon binding at the subcellular level and the active proteins at the binding site are fairly well characterized, then SAR approaches might work. An extensive study of ER binding affinity of a wide variety of steroidal and nonsteroidal ligands was conducted by Waller et al. (1996b). Fifty-five compounds were compared based on their steric and electrostatic properties. DES, selected estrogens, androgens, PCBs, OC insecticides, phthalates, and the hydroxylated metabolites of these compounds were all related to binding affinity in a statistically robust and internally consistent manner. The predictive limitations to this approach were due to the inconsistencies in the *in vitro* versus the *in vivo* systems used to generate the data (Jobling, 1998).

SARs can assist in identifying structural features common to a certain mode of action. The similarity of the structure of *o,p´*-DDT to DES was noted by (Bitman et al., 1968) when they reported the estrogenic activity of *o,p´*-DDT in animals. Most environmental estrogens possess a *para*-substituted phenolic group (Jordan et al., 1985). The presence of more than one phenolic group renders the compound more estrogenic (e.g., methoxychlor metabolites and diphenolic isoflavonoids). Structural rigidity also appears to be a predictor of estrogenic potency because of improved receptor binding. Conformationally restricted PCBs have a structure analogous to steroids (McKinney et al., 1994). Dodge (1998) reported that quantitative structure–activity studies for PCBs indicate that the electron density of the aromatic rings in PCB molecules correlate with binding affinity to the ER. Hydroxyl substitution on the phenyl ring of the PCB also provides stronger binding affinity to the ER (Korach et al., 1988). The concept of conformational restriction helps explain the fact that the *o,p´* isomer of DDT is estrogenic whereas the *p,p´*-DDT is much weaker.

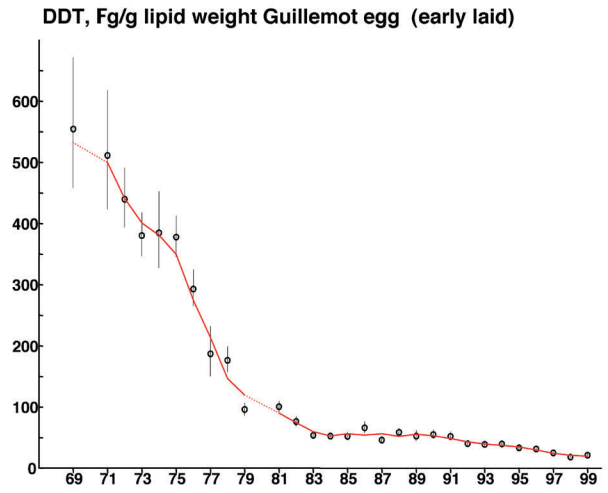
Ashby (1998) suggested that different SARs will be required for each mechanism of endocrine disruption. Currently, most SARs are based on ER interactions that relate to the chemical structure of E_2,

and predictability are poor for structurally remote analogues of E_2 (e.g., kepone and dieldrin) or testosterone (e.g., vinclozolin). Another drawback to the SAR approach is its failure to account for metabolic alterations that affect the activity of parent molecules. For example, *s*-methoprene becomes estrogenic after photolysis, forming a species that binds to the retinoic acid receptor (La Clair et al., 1998). A similar situation exist with vinclozolin, where *in vivo* activation produces metabolites that have androgen activity that would be missed with an *in vitro* test (Kelce et al., 1994).

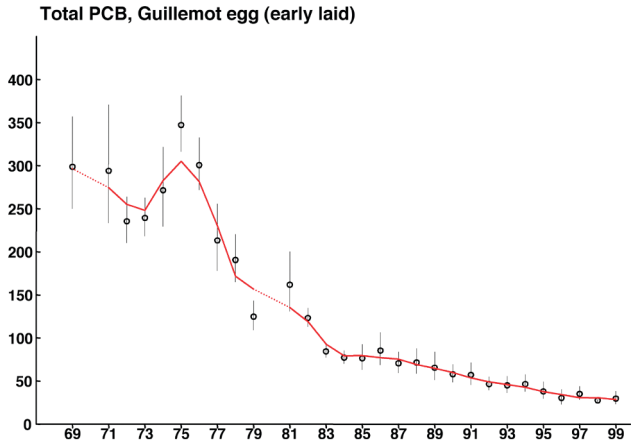
6.5 Summary

This chapter has illustrated the complexity and special problems related to measuring exposure to EDCs in wildlife and humans. The data clearly show that exposure to EDCs has occurred in wildlife species and human populations. However, except in isolated cases, data are not available to demonstrate specific associations between exposure and an endocrine-mediated adverse effect. Most exposure data focus on POPs in Europe and North America. Comparable data sets are not available for other nonpersistent EDCs in other geographical regions, making it difficult to make a truly global assessment. Existing exposure data sets relate primarily to external exposures (air, food, water) rather than internal exposure (blood, tissue), with the exception of POPs in breast milk and some human blood concentration data. It is unrealistic to monitor all potential EDCs in all species and matrices on a global scale. International, coordinated efforts and mechanisms need to be established to prioritize monitoring and collection of exposure data and to ensure comparability of data.

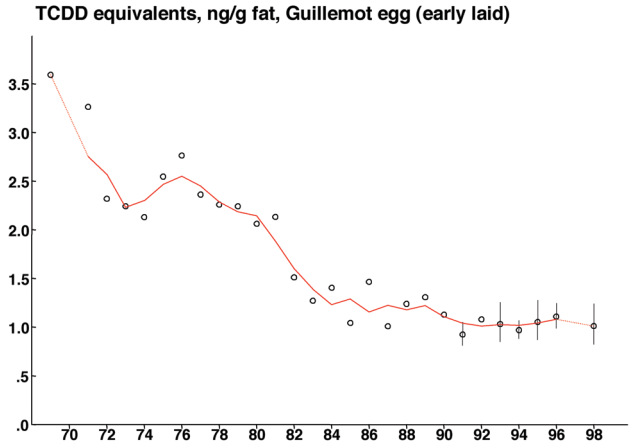
Chapter 6: Annex I



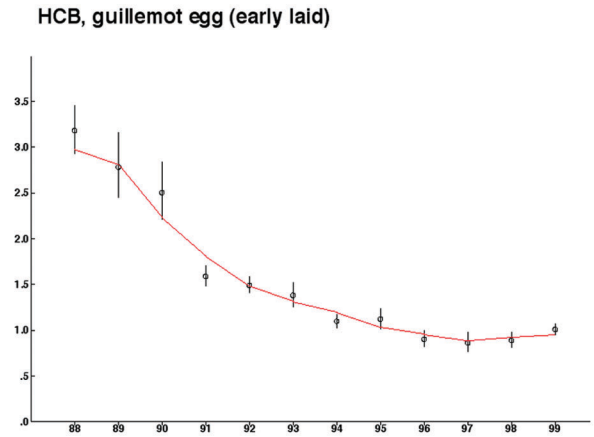
Annex I–Figure 1 - The temporal trend of total DDT (mainly DDE) in guillemot eggs from the central part of the Baltic studied during 1969–1998.



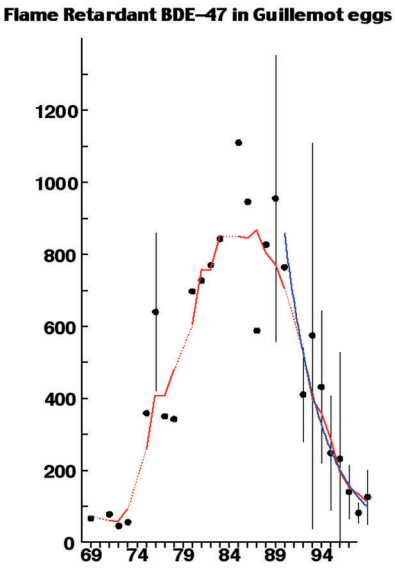
Annex I–Figure 2 - The temporal trend of total PCB in guillemot eggs from the central part of the Baltic studied during 1969–1998.



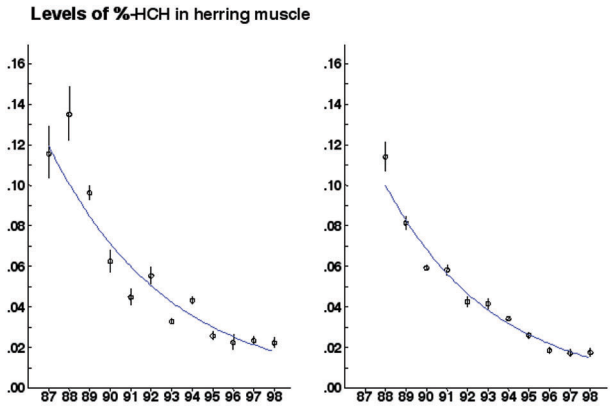
Annex I–Figure 3 - The temporal trend of PCDDs/PCDFs in guillemot eggs from the central part of the Baltic studied during 1969–1998.



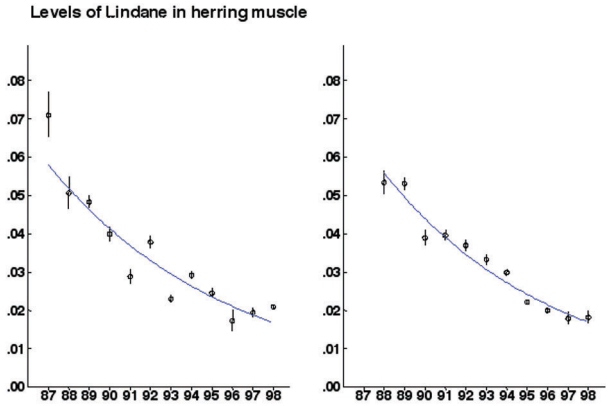
Annex I–Figure 4 - The temporal trend of HCB in guillemot eggs from the central part of the Baltic studied during 1988–1998.



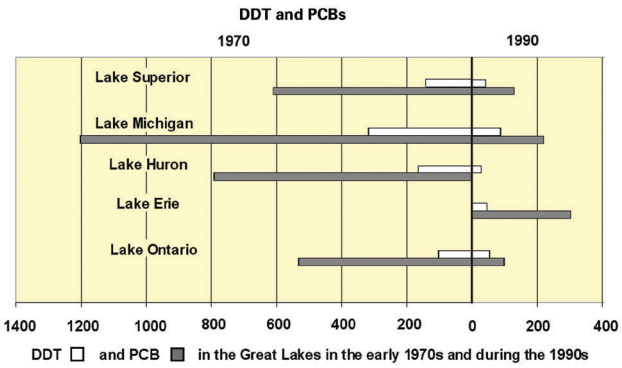
Annex I–Figure 5 - The temporal trend of the flame retardant BDE-47 in guillemot eggs from the central part of the Baltic studied during 1969–1998.



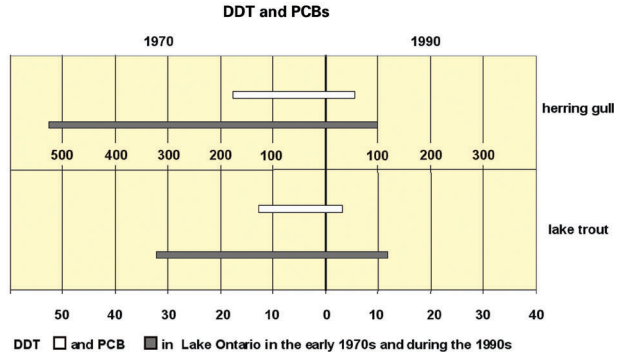
Annex I–Figure 6 - The temporal trend of α-HCH (µg/g fat) in herring from the central part of the Baltic studied during 1987–1998.



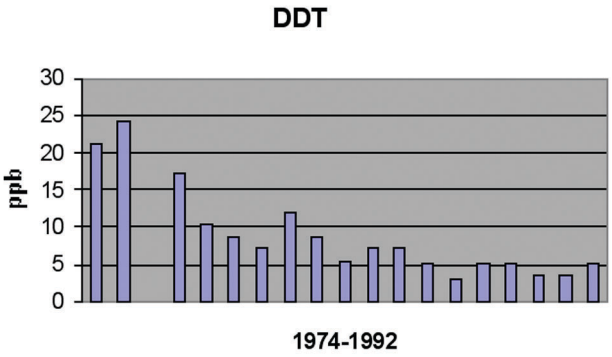
Annex I–Figure 7 - The temporal trend of γ-HCH (lindane; µg/g fat) in herring from the central part of the Baltic studied during 1987–1998.



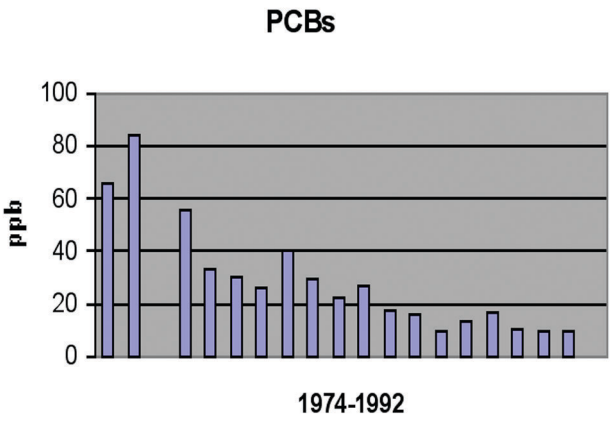
Annex I–Figure 8 - DDT and PCB concentrations in herring gulls from the Great Lakes in the 1970s and 1990s.



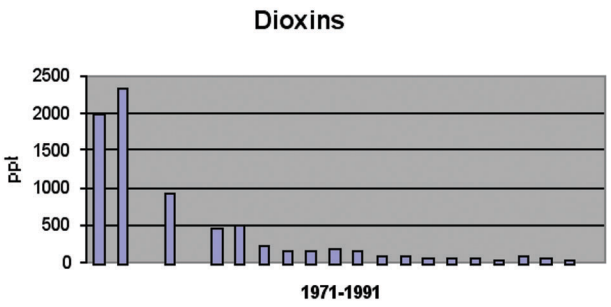
Annex I–Figure 9 - DDT and PCB concentrations in herring gulls and lake trout from Lake Ontario in the 1970s and 1990s.



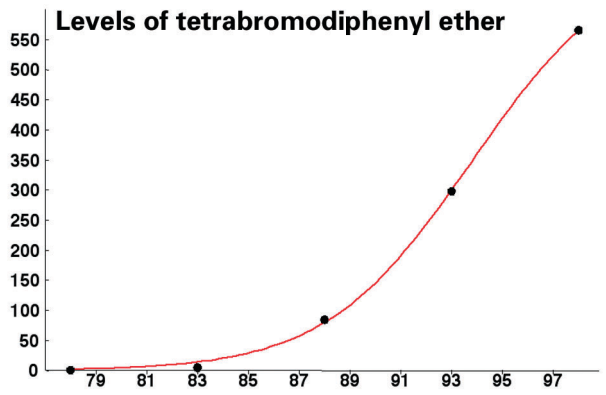
Annex I–Figure 10 - The temporal trend of concentrations of DDT compounds in eggs of herring gulls from Lake Ontario during 1974–1992.



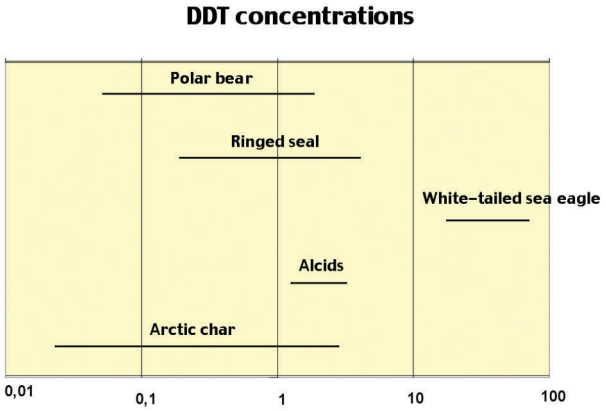
Annex I–Figure 11 - The temporal trend of concentrations of PCBs in eggs of herring gulls from Lake Ontario during 1974–1992.



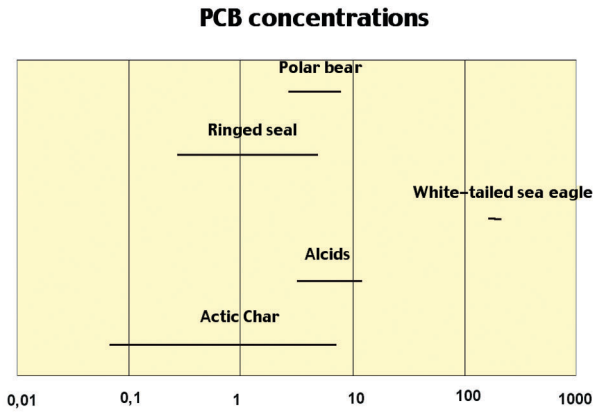
Annex I–Figure 12 - The temporal trend of dioxin concentrations in eggs of herring gulls from Lake Ontario during 1971–1991.



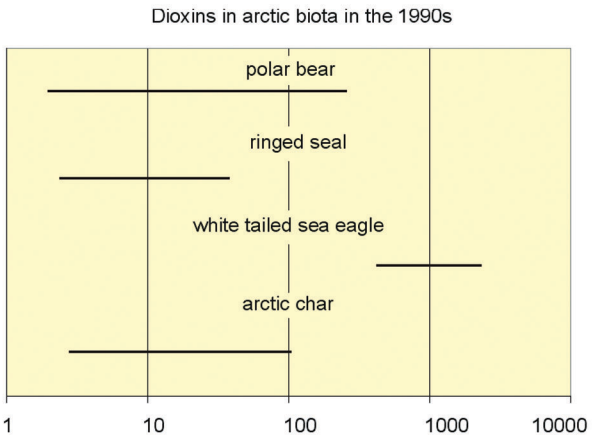
Annex I–Figure 13 - The temporal trend of 2,2´,4,4´-tetrabromodiphenyl ether levels in lake trout from Lake Ontario.



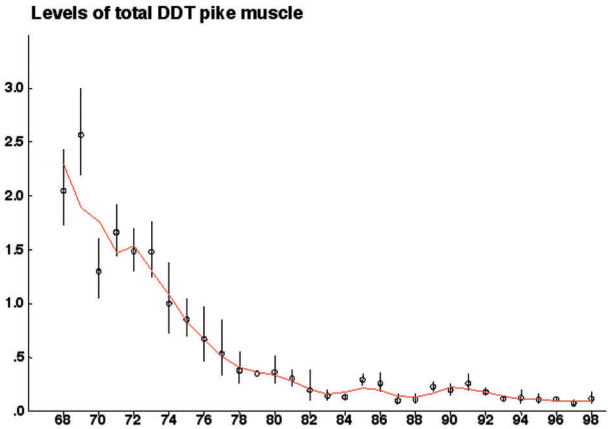
Annex I–Figure 14 - The range of DDT Concentrations in arctic biota in the 1990s.



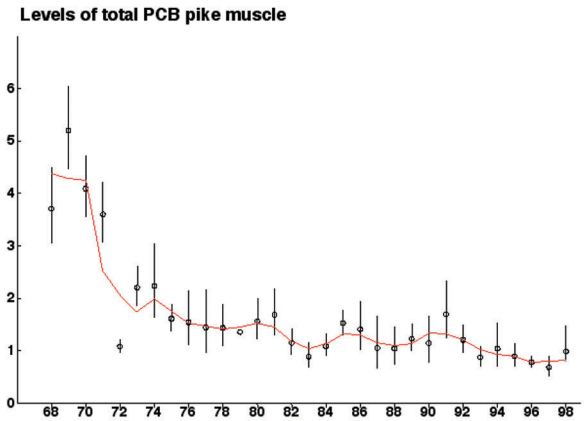
Annex I–Figure 15 - The range of PCB concentrations in arctic biota in the 1990s.



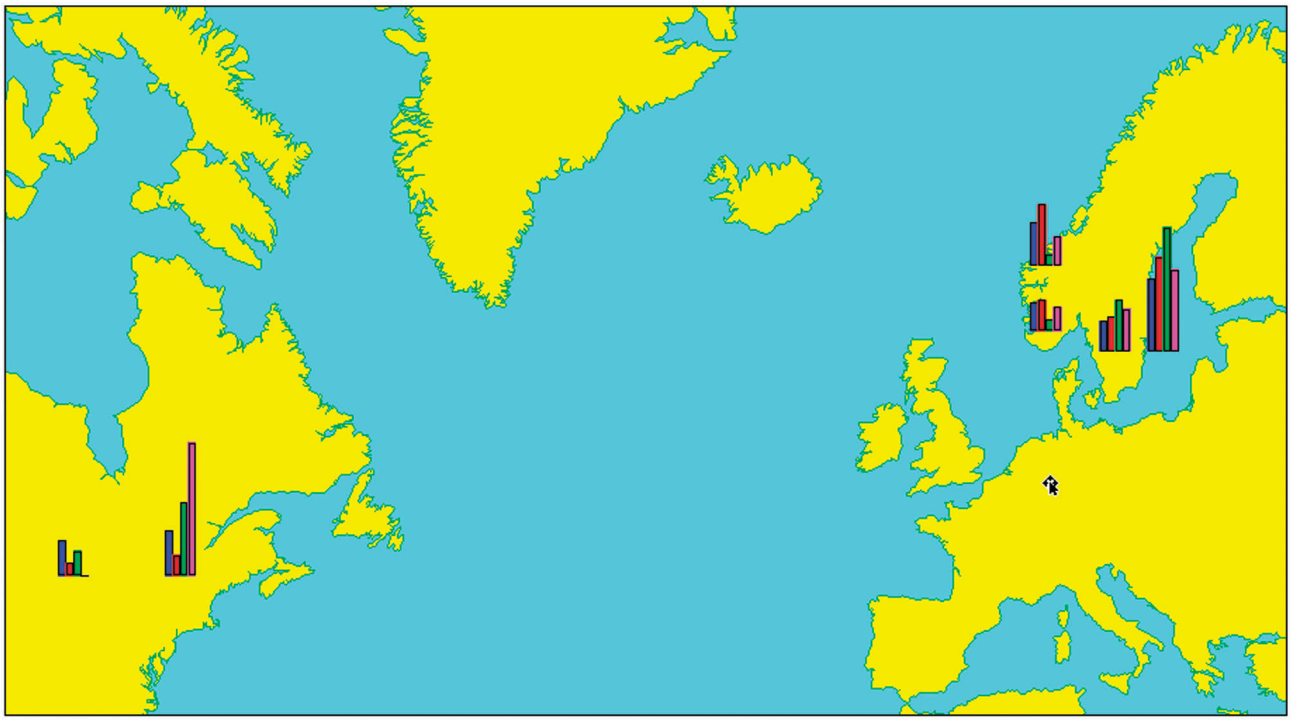
Annex I–Figure 16 - The range of PCDD/PCDF concentrations in arctic biota in the 1990s.



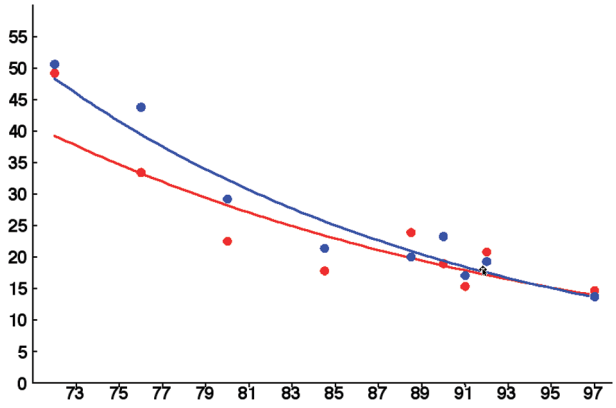
Annex I–Figure 17 - The temporal trend of DDT in pike muscle (µg/g fat) from Lake Storvindeln in subarctic Sweden during 1968–1998.



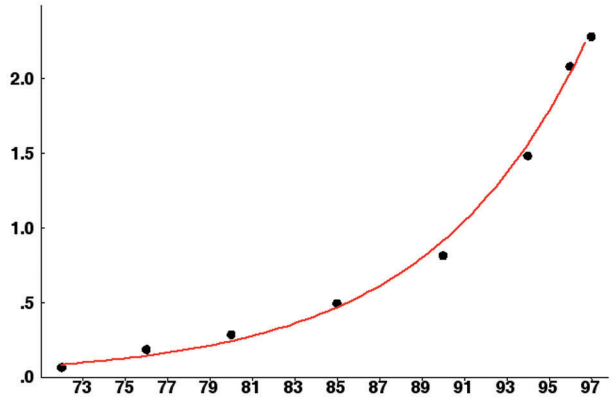
Annex I–Figure 18 - The temporal trend of PCB in pike muscle (µg/g fat) from Lake Storvindeln in subarctic Sweden during 1968–1998.



Annex I–Figure 19 - TEQs and PCBs in fisheaters.



Annex I–Figure 20 - Time trend of TEQs in mothers' milk (pg/g fat) from Sweden.



Annex I–Figure 21 - Time trend of BDE-47 concentrations (ng/g fat) in Swedish mothers' milk.

Annex I–Table 1 - Total DDT (µg/g lipid weight) in Wildlife Species from the Baltic Sea Area

Species	Collection Year	*N*	Mean	95% CI	Range	Reference (First author, year)
Herring						
Muscle	1969–1970	267	46 (a)[a]		6.3–250	Jensen, 1972
Muscle	1970	24	57 (a)			Andersson, 1988a
Muscle[b]	1972	370	18 (m)[c]	14–23		Bignert, 1998b
Muscle	1978	40	6.8 (a)			Jansson, 1979
Muscle	1978–1982	15	4.6 (a)		1.7–8.9	Moilanen, 1982
Muscle	1979	25	2.3 (a)			Andersson, 1988a
Muscle[d]	1980	94	1.4 (m)	1.1–1.8		Bignert, 1998b
Muscle	1986	100[e]	0.43 (a)		0.32–0.54	Haahti, 1988
Muscle[b]	1992	19	0.28 (a)		0.10–0.58	Roots, 1995
Muscle[b]	1995	370	1.1 (m)	0.84–1.4		Bignert, 1998b
Muscle[d]	1995	94	0.53 (m)	0.41–0.70		Bignert, 1998b
Salmon						
Muscle	1970	34	29 (a)		10–60	Jensen, 1972
Muscle	1971	14	20 (a)			Andersson, 1988a
Muscle	1979	3	24 (a)		6.3–8.8	Andersson, 1988a
Muscle	1988–1992	73	5.4 (a)	4.0	1.3–28	Vuorinen, 1997a
Muscle	1995	31	3.4 (a)	2.5–5.2		Asplund, 1999
Egg	1995	31	1.8 (a)		1.1–2.6	Asplund, 1999
Blood	1995	31	1.0 (a)		0.6–2.0	Asplund, 1999
Guillemot						
Breast muscle	1970–1981	19[c]	250 (a)		74–400	Andersson, 1988a
Egg	1969	211	512 (m)	390–670		Bignert, 1998b
Egg	1996	211	25 (m)	19–32		Bignert, 1998b
Cormorant						
Breast muscle	1979	4	64 (a)			Andersson, 1988a
White-tailed sea eagle						
Egg	1965–1978	34	825 (a)	375	245–1,900	Helander, 1982
Egg	1979	2	835 (a)			Andersson, 1988a
Egg	1995–1997	5	110 (a)		70–160	Helander, 1998
Gray seal						
Blubber, pups	1969–1973	23	250 (g)[f]		70–979	Blomkvist, 1992
Blubber, pups	1981–1988	10	35 (g)		19–91	Blomkvist, 1992
Blubber, pups	1995–1997	13	12 (g)			Roos, 1998
Blubber, adults	1969–1972	60	300 (a)		68–970	Olsson, 1975
Blubber, adults	1976–1982	9	42 (a)		6.4–92	Perttilä, 1986
Blubber, adults	1980–1990	15	70 (a)		11–180	Blomkvist, 1992
Ringed seal						
Blubber	1969–1972	33	200 (a)		31–770	Olsson, 1975
Blubber	1976–1982	19	76 (a)		9–161	Perttilä, 1986
Blubber, adults	1980–1986	7	230 (a)		150–820	Blomkvist, 1992
Harbor seal						
Blubber, pups	1983–1989	17	23 (a)		12–60	Blomkvist, 1992

[a]Arithmetic. [b]Spring collected. [c]Median. [d]Autumn collected. [e]In five pools. [f]Geometric.

Annex I–Table 2 - Total PCB (µg/g lipid weight) in Wildlife Species from the Baltic Sea Area

Species	Collection Year	*N*	Mean	95% CI	Range	Reference (First author, year)
Herring						
Muscle	1969–1970	267	17 (a)[a]		2.4–110	Jensen, 1972
Muscle	1970	24	24 (a)			Andersson, 1988a
Muscle[b]	1972	370	16 (m)[c]	12–21		Bignert, 1998b
Muscle	1979	25	2.5 (a)			Andersson, 1988a
Muscle	1978–1982	15	11 (a)		3.0–19.2	Moilanen, 1982
Muscle	1978	40	7.9 (a)			Jansson, 1979
Muscle[d]	1980	94	2.2 (m)	1.7–2.9		Bignert, 1998b
Muscle	1986	100[e]	0.94 (a)		0.76–1.1	Haahti, 1988
Muscle[b]	1996	370	2.4 (m)	1.8–3.2		Bignert, 1998b
Muscle[d]	1996	94	1.1 (m)	0.87–1.5		Bignert, 1998b
Salmon						
Muscle	1970	34	12 (a)		5.8–25	Jensen, 1972
Muscle	1971	14	8.4 (a)			Andersson, 1988a
Muscle	1979	5	10 (a)			Andersson, 1988a
Muscle	1988–1992	73	5.0 (a)	2.83	1.2–21	Vuorinen, 1997
Muscle	1995	31	8.0 (a)		5.2–11	Asplund, 1999
Egg	1995	31	3.8 (a)		2.4–5.0	Asplund, 1999
Blood	1995	31	4.6 (a)		2.7–7.2	Asplund, 1999
Muscle	1996	50	2.3 (m)		0.5–4.5	Atuma, 1998
Guillemot						
Breast muscle	1970–1981	19[e]	210 (a)		100–310	Andersson, 1988a
Egg	1969	211	380 (m)	330–450		Bignert, 1998b
Egg	1996	211	37 (m)	32–43		Bignert, 1998b
Cormorant						
Breast muscle	1979	4	130 (a)			Andersson, 1988a
Breast muscle	1992	3	49 (a)	31		Bignert, 1998b
White-tailed sea eagle						
Egg	1979	2	775 (a)			Andersson, 1988a
Egg	1965–1978	34	1,100 (a)	445	260–2,200	Helander, 1982
Egg	1995–1997	5	390 (a)		260–590	Helander, 1999
Gray seal						
Blubber, pups	1969–1973	23	93 (g)[f]		21–290	Blomkvist, 1992
Blubber, pups	1981–1988	10	77 (g)		32–110	Blomkvist, 1992
Blubber, pups	1995–1997	13	38 (g)			Roos, 1998
Blubber	1969–1972	60	112		20–330	Olsson, 1975
Blubber	1976–1982	9	53 (a)		26–112	Perttilä, 1986
Blubber, adults	1980–1990	15	190 (a)		57–770	Blomkvist, 1992
Ringed seal						
Blubber	1969–1972	33	110 (a)		27–390	Olsson, 1975
Blubber	1976–1982	19	76 (a)			Perttilä, 1986
Blubber, adults	1980–1986	7	210 (g)		120–770	Blomkvist, 1992
Harbor seal						
Blubber, pups	1983–1989	17	33 (a)		16–98	Blomkvist, 1992

[a] Arithmetic. [b]Spring collected. [c]Median. [d]Autumn collected. [e]In five pools. [f]Geometric.

Annex I–Table 3 - HCB (µg/g lipid weight) in Wildlife Species from the Baltic Sea Area

Species	Collection Year	*N*	Mean	95% CI	Range	Reference (First author, year)
Herring						
Muscle	1986	100[a]	0.016 (a)[b]		0.014–0.018	Haahti, 1988
Muscle[c]	1988	160	0.10 (m)[d]	0.078–0.13		Bignert, 1998b
Muscle[e]	1988	160	0.20 (m)	0.14–0.29		Bignert, 1998b
Muscle	1992	19	0.018 (a)		0.006–0.042	Roots, 1995
Muscle[c]	1995	160	0.025 (m)	0.019–0.033		Bignert, 1998b
Muscle[e]	1995	160	0.067 (m)	0.049–0.092		Bignert, 1998b
Salmon						
Muscle	1988–1992	73	0.17 (a)	0.066	0.068–0.36	Vuorinen, 1997a
Muscle	1995	33	0.092 (a)	21	0.062–0.130	Asplund, 1999
Egg	1995	33	0.10 (a)		0.044–0.150	Asplund, 1999
Blood	1995	33	0.031 (a)		0.008–0.058	Asplund, 1999
Guillemot						
Egg	1988	80	5.7 (m)	3.6–8.9		Bignert, 1998b
Egg	1995	80	1.2 (m)			Bignert, 1998b
Egg	1969	10	4.4 (a)			Bignert, 1998b

[a]In five pools. [b]Arithmetic. [c]Autumn collected. [d]Median. [e]Spring collected.

Annex I–Table 4 - α-HCH (µg/g lipid weight) in Wildlife Species from the Baltic Sea Area

Species	Collection Year	*N*	Mean	95% CI	Range	Reference (First author, year)
Herring						
Muscle[a]	1988	160	0.096 (m)[b]	0.076–0.12		Bignert, 1998b
Muscle[c]	1988	160	0.12 (m)	0.10–0.15		Bignert, 1998b
Muscle	1992	16	0.009 (a)[d]		0.0006–0.026	Roots, 1995
Muscle[a]	1995	160	0.026 (m)	0.020–0.032		Bignert, 1998b
Muscle[c]	1995	160	0.028 (m)	0.024–0.028		Bignert, 1998b
Salmon						
Muscle	1988–1992	73	0.011 (a)		ND-0.38	Vuorinen, 1997a
Guillemot						
Egg	1988	80	0.095 (m)	0.060–0.15		Bignert, 1998b
Egg	1995	80	0.016 (m)	0.010–0.025		Bignert, 1998b

[a]Autumn collected. [b]Median. [c]Spring collected. [d]Arithmetic.

Annex I–Table 5 - β-HCH (µg/g lipid weight) in Wildlife Species from the Baltic Sea Area

Species	Collection Year	*N*	Mean	95% CI	Range	Reference (First author, year)
Herring						
Muscle[a]	1988	160	0.048 (m)[b]	0.038–0.061		Bignert, 1998b
Muscle[c]	1988	160	0.047 (m)	0.036–0.063		Bignert, 1998b
Muscle	1992	6	0.014 (a)[d]		0.008–0.030	Roots, 1995
Muscle[a]	1995	160	0.021 (m)	0.017–0.027		Bignert, 1998b
Muscle[c]	1995	160	0.021 (m)	0.017–0.027		Bignert, 1998b
Guillemot						
Egg	1969	80	1.2 (m)	0.950–1.60		Bignert, 1998b
Egg	1996	80	0.46 (m)	0.36–0.59		Bignert, 1998b

[a]Autumn collected. [b]Median. [c]Spring collected. [d]Arithmetic.

Annex I–Table 6 - Lindane (µg/g lipid weight) in Wildlife Species from the Baltic Sea Area

Species	Collection Year	*N*	Mean	95% CI	Range	Reference (First author, year)
Herring						
Muscle	1986	100[a]	0.015 (a)[b]		0.011–0.019	Haahti, 1988
Muscle[c]	1988	160	0.055 (m)[d]	0.048–0.064		Bignert, 1998b
Muscle[e]	1988	160	0.088 (m)	0.070–0.10		Bignert, 1998b
Muscle	1992	16	0.010 (a)		0.001–0.026	Roots, 1995
Muscle[c]	1995	160	0.025 (m)	0.022–0.029		Bignert, 1998b
Muscle[e]	1995	160	0.024 (m)	0.020–0.030		Bignert, 1998b
Salmon						
Muscle	1988–1992	73	0.012 (a)	0.013	n.d–0.075	Vuorinen, 1997a
Guillemot						
Egg	1988	80	0.064 (m)	0.029–0.14		Bignert, 1998b
Egg	1995	80	0.010 (m)	0.004–0.022		Bignert, 1998b

[a]In five pools. [b]Arithmetic. [c]Autumn collected. [d]Median. [e]Spring collected.

Annex I–Table 7 - Chlordanes (µg/g lipid weight) in Wildlife Species from the Baltic Sea Area

Species	Collection Year	*N*	Mean	Range	Reference (First author, year)
Herring					
Muscle	1970	24	0.52 (a)[a]		Andersson, 1988a
Muscle	1978–1982	15	0.48 (a)	0.21–0.83	Moilanen, 1982
Muscle	1979	25	0.20 (a)		Andersson, 1988a
Muscle	1978	40	0.6 (a)		Jansson, 1979
Salmon					
Muscle	1971	14	0.28 (a)		Andersson, 1988a
Muscle	1979	3	0.34 (a)	0.30–0.39	Andersson, 1988a
Muscle	1988–1992	73	0.36 (a)	0.18–0.99	Vuorinen, 1997a
Guillemot					
Egg	1978	10	0.7 (a)		Jansson, 1979
		10	3.4 (a)		
Gray seal					
Adults	1974–1978	5	10 (a)		Jansson, 1979
Adults	1980–1990	23	16 (a)	3.3–54	Andersson, 1992
Pups	1980–1990	10	3.4 (a)		Andersson, 1992
Ringed seal					
Adults	1980–1986	5	11 (a)		Andersson, 1992
Harbor seal					
Pups	1983–1989	17	1.4 (a)	0.8–1.8	Andersson, 1992

[a]Arithmetic.

Annex I–Table 8 - PCBs (µg/g lipid weight) in Wildlife Species from the Baltic Sea Area

Species	Collection Year	*N*	Mean	Range	Reference (First author, year)
Herring					
Muscle	1979	25	3.9 (a)[a]		Andersson, 1988a
Muscle	1970	24	14 (a)		Andersson, 1988a
Muscle	1978	40	13 (a)		Jansson, 1979
Salmon					
Muscle	1971	14	6.5 (a)		Andersson, 1988a
Muscle	1979	3	5.7 (a)	5.2–6.2	Andersson, 1988a
Muscle	1988–1992	73	1.42 (a)	1.2–21	Vuorinen, 1997a
Guillemot					
Egg	1974–1976	10	70 (a)		Wideqvist, 1993
Egg	1987–1989	10	26 (a)		Wideqvist, 1993
Egg	1978	10	17 (a)		Jansson, 1979
Breast muscle	1970–1981	19	6.0 (a)	4.2–7.7	Andersson, 1988a
Cormorant					
Breast muscle	1975	4	1.7 (a)		Andersson, 1988a
White-tailed sea eagle					
Egg	1965	2	8.5 (a)		Andersson, 1988a
Gray seal					
Pups	1981–1988	10	3.8 (a)		Wideqvist, 1993
Pups	1976–1978	2	5.8 (a)		Andersson, 1988a
Adults	1980–1990	23	3.3 (a)	1.9–10	Wideqvist, 1993
Adult	1974–1977	5	11		Jansson, 1979
Ringed seal					
Adult males	1980–1986	5	14 (a)		Wideqvist, 1993
Adult	1981	1	12		Andersson, 1988a
Harbor seal					
Pups	1983–1989	17	2.4 (a)	1.4–3.6	Wideqvist, 1993

[a]Arithmetic.

Annex I–Table 9 - PBDE (µg/g lipid weight) in Wildlife Species from the Baltic Sea Area

Species	Collection Year	*N*	Mean	Range	Reference (First author, year)
Herring					
Muscle, spring collected[a]	1987	60	0.530 (a)[b]		Sellström, et al., 1993
Muscle, autumn collected[c]	1987	10	0.047 (a)	0.017–0.055	Sellström, et al., 1993
Salmon					
Muscle	1995	31	0.290 (a)	0.190–0.510	Asplund, 1999
Egg	1995	31	0.99 (a)	0.075–0.150	Asplund, 1999
Blood	1995	31	0.30 (a)	0.130–0.630	Asplund, 1999
Guillemot					
Guillemot, egg	1974	10	0.230 (a)		Sellström et al., 1993
Guillemot, egg	1989	10	1.200 (a)		Sellström et al., 1993
Gray seal					
Blubber, pups	1981–1988	10	0.30 (a)		Andersson, 1992
Blubber, adults	1980–1990	11	0.33 (a)		Andersson, 1992
Blubber	1979–1985	8	0.73 (a)		Sellström et al., 1993
Ringed seal					
Blubber, adults	1980–1986	5	0.32 (a)		Andersson, 1992
Harbor seal					
Blubber, pups	1983–1989	17	0.56 (a)		Andersson, 1992

[a]Spring collected. [b]Arithmetic. [c]Autumn collected.

Annex I–Table 10 - Dioxin-TEQ (pg/g lipid weight) in Wildlife Species from the Baltic Sea Area

Species	Collection Year	*N*	Mean	Range	Reference (First author, year)
Herring, muscle					
Northern Baltic	1994, 2 years of age	10	17		de Wit, 1999
Northern Baltic	1988–1993, 2 years	20	34	31–37	de Wit, 1994
Southern Baltic	1994, 2 years	10	22		de Wit, 1999
Southern Baltic	1988–1993, 2 years	10	23		de Wit, 1994
Southern Baltic	1988–1993, 2 years	10	26		de Wit, 1994
Southern Baltic	1988–1993, 4 years	10	60		de Wit, 1994
Southern Baltic	1988–1993, 6 years	10	74		de Wit, 1994
Guillemot egg					
1969–1979		100	2,600	2,130–3,600	
1988–1994		70	1,100	970–1,300	
White-tailed sea eagle					
Breast muscle			2700		de Wit, 1994
Gray seal, blubber					
Juveniles	1987	10	15		Bignert, 1989
Juveniles	1985–1990	10	23		Bergek, 1992
Adult males	1985–1990	5	14		Bergek, 1992
Adult females	1985–1990	18	17		Bergek, 1992
Ringed seal, blubber					
Juveniles	1986–1987	5	122		Bignert, 1989
Juveniles	1985–1990	10	67		Bergek, 1992
Adults	1986–1987	5	59		Bignert, 1989
Adult males	1985–1990	5	166		Bergek, 1992
Harbor seal, blubber					
Juveniles	1983–1987	9	12		Bignert, 1989
Juveniles	1985–1990	14	14		Bergek, 1992

Annex I–Table 11 - Levels of Total DDT (µg/g lipid weight) in the Great Lakes Area

Species, Lake	Collection Year	*N*	Mean Σ DDT	Mean DDE	S.D.	95% CI	Reference (First author, year)
Lake trout							
Michigan, whole body	1972	9	61.1 (a)[a]			43.5–78.8	De Vault, 1986
Michigan, whole body	1973	30	62.3 (a)			53.8–131.3	De Vault, 1986
Michigan, whole body	1977	15[b]	38.1 (a)			–2.4–78.9	De Vault, 1986
Michigan, whole body	1978	40[c]	26.2 (a)			13.3–39.0	De Vault, 1986
Michigan, whole body	1979	30[d]	41.1 (a)			16.7–65.5	De Vault, 1986
Ontario, whole body	1977	48		11.8 (g)[e]			Borgmann, 1991
Ontario, whole body	1978	141		5.9 (g)			Borgmann, 1991
Ontario, whole body	1979	176		6.3 (g)			Borgmann, 1991
Ontario, whole body	1977	6		8.6 (a)	3.5		Huestis, 1996
Ontario, whole body	1978	7		4.6 (a)	1.6		Huestis, 1996
Huron, whole body	1978	6	11.4 (a)			10.6–12.1	De Vault, 1986
Huron, whole body	1979	5	13.4 (a)			9.1–17.7	De Vault, 1986
Superior, whole body	1977	7	7.3 (a)			3.1–6.6	De Vault, 1986
Superior, whole body	1979	6	5.0 (a)			2.8–7.2	De Vault, 1986
Ontario, whole body	1992	7		3.7 (a)	0.82		Huestis, 1996
Ontario, whole body	1993	7		5.5 (a)	0.98		Huestis, 1996
Herring gull							
Michigan, egg	1977			320 (a)			Tillitt, 1998
Michigan, egg	1978			240 (a)			Tillitt, 1998
Michigan, egg	1991			110 (a)			Tillitt, 1998
Michigan, egg	1992			90 (a)			Tillitt, 1998
Ontario, egg	1977			170 (a)			Tillitt, 1998
Ontario, egg	1978			120 (a)			Tillitt, 1998
Ontario, egg	1991			33 (a)			Tillitt, 1998
Ontario, egg	1992			55 (a)			Tillitt, 1998
Huron, egg	1977			170 (a)			Tillitt, 1998
Huron, egg	1978			72 (a)			Tillitt, 1998
Huron, egg	1991			28 (a)			Tillitt, 1998
Huron, egg	1992			33 (a)			Tillitt, 1998
Superior, egg	1977			130 (a)			Tillitt, 1998
Superior, egg	1978			110 (a)			Tillitt, 1998
Superior, egg	1991			39 (a)			Tillitt, 1998
Superior, egg	1992			44 (a)			Tillitt, 1998
Double-crested cormorant							
Ontario, egg	1970–1972	7		271 (a)	63		Ryckman, 1998
Ontario, egg	1981	20		113 (a)	54		Ryckman, 1998
Ontario, egg	1995	30.f		55			Ryckman, 1998
Huron, egg	1970–1972	21		258 (a)	135		Ryckman, 1998
Huron, egg	1979	9		54 (a)	62		Ryckman, 1998
Huron, egg	1995	10.f		43			Ryckman, 1998
Superior, egg	1983	10.f		52			Ryckman, 1998
Superior, egg	1995	10.f		57			Ryckman, 1998
Erie, egg	1970–1972	18		77 (a)	56		Ryckman, 1998
Erie, egg	1979	10		109 (a)	56		Ryckman, 1998
Erie, egg	1995	10.f		47			Ryckman, 1998
Mink							
Erie, Mersea, liver	1988–1989	9		1.4 (a)	0.4		Haffner, 1998
Erie, Dover, liver	1988–1989	9		0.76 (a)	0.2		Haffner, 1998
Erie, Dorchester, liver	1988–1989	1		0.16 (a)			
Ontario, Darlington, liver	1988–1989	2			1.8 (a)	1.3	

[a]Arithmetic mean. [b]In 3 pools. [c]In eight pools. [d]In six pools. [e]Geometric mean. [f]In one pool.

Annex I–Table 12 - Levels of Total PCBs (µg/g lipid weight) in the Great Lakes Area

Species, Lake	Collection Year	*N*	Mean PCB	95% CI	Reference (First author, year)
Lake trout					
Michigan, whole body	1972	9	69.5	43.8–95.2	De Vault, 1986
Michigan, whole body	1973	30	118	105.3–131.3	De Vault, 1986
Michigan, whole body	1977	15[a]	69.8 (a)[b]	–13.9–153.0	De Vault, 1986
Michigan, whole body	1978	40[c]	46.7 (a)	31.4–62.2	De Vault, 1986
Michigan, whole body	1979	30[d]	52.5 (a)	30.9–74.4	De Vault, 1986
Ontario, whole body	1977	48	31.7 (g)[e]		Borgmann, 1991
Ontario, whole body	1978	141	40.9 (g)		Borgmann, 1991
Ontario, whole body	1979	176	19.5 (g)		Borgmann, 1991
Ontario, whole body	1977	6	35.3 (a)		Huestis, 1996
Ontario, whole body	1978	7	25.8 (a)		Huestis, 1996
Ontario, whole body	1992	7	11.5 (a)		Huestis, 1996
Ontario, whole body	1993	7	13.9 (a)		Huestis, 1996
Huron, whole body	1978	6	15.2 (a)	12.1–18.3	De Vault, 1986
Huron, whole body	1979	5	17.2 (a)	7.4–27.0	De Vault, 1986
Superior, whole body	1977	7	11.3 (a)	5.8–17.0	De Vault, 1986
Superior, whole body	1978	7	4.4 (a)	3.1–5.7	De Vault, 1986
Superior, whole body	1979	6	4.0 (a)	2.7–5.4	De Vault, 1986
Herring gull					
Michigan, egg	1977		1,200 (a)		Tillitt, 1998
Michigan, egg	1978		1,000 (a)		Tillitt, 1998
Michigan, egg	1991		340 (a)		Tillitt, 1998
Michigan, egg	1992		220 (a)		Tillitt, 1998
Ontario, egg	1977		530 (a)		Tillitt, 1998
Ontario, egg	1978		390 (a)		Tillitt, 1998
Ontario, egg	1981	9	538 (a)		Haffner, 1997
Ontario, egg	1991		79 (a)		Tillitt, 1998
Ontario, egg	1992		95 (a)		Tillitt, 1998
Ontario, egg	1992	10	166 (a)		Haffner, 1997
Huron, egg	1977		790 (a)		Tillitt, 1998
Huron, egg	1978		330 (a)		Tillitt, 1998
Huron, egg	1991		110 (a)		Tillitt, 1998
Huron, egg	1992		110 (a)		Tillitt, 1998
Superior, egg	1977		610 (a)		Tillitt, 1998
Superior, egg	1978		470 (a)		Tillitt, 1998
Superior, egg	1991		150 (a)		Tillitt, 1998
Superior, egg	1992		140 (a)		Tillitt, 1998
Erie, egg	1981	10	399 (a)		Haffner, 1997
Erie, egg	1992	10	320 (a)		Haffner, 1997
Double-crested cormorant					
Ontario, egg	1970–1972	7	529 (a)		Ryckman, 1998
Ontario, egg	1981	20	600 (a)		Ryckman, 1998
Ontario, egg	1981	10	281 (a)		Haffner, 1997
Ontario, egg	1992	10	230 (a)		Haffner, 1997
Ontario, egg	1995	30[f]	121		Ryckman, 1998
Huron, egg	1970–1972	9	393 (a)		Ryckman, 1998
Huron, egg	1995	10[f]	110		Ryckman, 1998
Superior, egg	1983	10[f]	213		Ryckman, 1998
Superior, egg	1995	10[f]	50		Ryckman, 1998
Erie, egg	1970–1972	18	228 (a)		Ryckman, 1998
Erie, egg	1979	10	856 (a)		Ryckman, 1998
Erie, egg	1981	10	670 (a)		Haffner, 1997
Erie, egg	1992	10	409 (a)		Haffner, 1997
Erie, egg	1995	10[f]	303		Ryckman, 1998
Mink					
Erie, Mersea, liver	1988–1989	9	23.3 (a)		Haffner, 1998
Erie, Dover, liver	1988–1989	9	13.8 (a)		Haffner, 1998
Erie, Dorchester, liver	1988–1989	1	14.1		
Ontario, Darlington, liver	1988–1989	2	13.0 (a)		

[a] In tree pools. [b] Arithmetic mean. [c] In eight pools. [d] In six pools. [e] Geometric mean. [f] In one pool.

Annex I–Table 13 - Total DDT levels (µg/g lipid weight) in Arctic Species

Species	Range
Arctic char muscle	0.023–2.75
Mustelids	0.014–0.98
Glaucous gull, eggs	2.84–18.3
Alcids, eggs	1.27–3.2
White-tailed sea eagle, eggs	17.5–70.0 (*p,p´*-DDE)
Ringed seal, blubber	0.19–4.0
Polar bear, fat	0.052–1.82 (*p,p´*-DDE)

Source: de March et al. (1998).

Annex I–Table 14 - Total PCB Levels (µg/g lipid weight) in Arctic Species

Species	Range
Arctic char muscle	0.069–7.0
Mustelids	0.093–8.0
Glaucous gull, eggs	4.6–26.6
Alcids, eggs	3.2–12.0
White-tailed sea eagle, eggs	170–208
Ringed seal, blubber	0.27–4.7
Polar bear, fat	2.76–80.3

Source: de March et al. (1998).

Annex I–Table 15 - Levels of TCDD Equivalents (pg/g lipid weight) in Arctic Species

Species	Range
Arctic char muscle	2.8–103
Mustelids	100–270
White-tailed sea eagle, eggs	425–2,300
Ringed seal, blubber	2.4–38
Polar bear, fat	2–256

Source: de March et al. (1998).

Annex I–Table 16 - Total DDT and PCB Levels (µg/g lipid weight) in Blubber of Fish-Eating Marine Mammals and Polar Bears

Continent/ Water	Country	Locality	Species	*n*	Collection Year	DDT	PCB	Reference (First author, year)
Europe								
	UK	The Wash	Common seal juv.	16	1988	2.9	12.5	Hall, 1992
		S. Lough		15	1988	0.69	36	Hall, 1992
		M. Firth		26	1988	1.92	11.8	Hall, 1992
		W. Coast		16	1988	2.15	15.5	Hall, 1992
		Orkney		16	1988	1.2	10.5	Hall, 1992
	Ireland	Northern coast		44	1988	2,6	26	Mitchell, 1992
	Sweden	The Baltic	Common seal pups	17	1980–1990	28.4	58	Blomkvist, 1992
			Gray seal adults	15	1980–1990	55	140	
			Ringed seal adults	7	1980–1990	230	210	
	Spain	Mediterranean	Striped dolphin	72	1987–1991		300	Aguilar, 1994
North America								
	Canada	Eastern Canada	Gray seal	8	1982	3.5	16	Schröter-Kermani, 2000
		St. Lawrence Estuary	Gray seal	5	1989–1996	2.4	10	Bernt, 1999
		St. Lawrence Estuary	Harbor seal	17	1989–1996	5.0	26	Bernt, 1999
Arctic								
	Baffin Bay		Beluga	208	1983–1989	2.8	3.8	Norstrom, 1994
			Narwhal	21	1982–1983	4.9	4.5	
			Ringed seal	202	1983–1988	0.51	0.60	
			Polar bear	121	1982–1984	0.40	5.4	
	Norway	Spitzbergen	Ringed seal	20	1986–1990	1.49	1.68	Norstrom, 1994
		Spitzbergen	Polar bear	10	1978–1989	0.92	26.2	
	Canada	NWT	Ringed seal	28	1981	0.56	0.94	Addison, 1986
	Bering Sea		Fur seal	37	1981–1987	3.0	1.94	Norstrom, 1994
Pacific								
	Japan							
		Northern Pacific	Northern fur seal	5	1986	1.7	4.0	Tanabe, 1994
		Northern Pacific	Larga seal	4	1991	17	23	Tanabe, 1994
		Northern Pacific	Dall's porpoise	4	1983	13	19	Tanabe, 1994
		Northern Pacific	Stellar sea lion	4	1990	10	19	Tanabe, 1994
Africa								
	South Africa		Common dolphin	17	1984–1987	5.4	4.1	De Kock, 1994

NWT, Northwest Territories; UK, United Kingdom.

Annex I–Table 17 - Levels of Mono-, Di-, and Tributyl Tins in Japan

Sample Type	*N*	MBT	DBT	TBT	BT	Unit	Reference (First author, year)
Seawater	7	<8.0–11	<4.6–8.1	<3.0–19	<15–27	ng/liter	Takahashi, 1999
Sediment	2				70–80	ng/g d.w.	Takahashi, 1999
Caprellids, whole body		11–28	7.9–17	57–140	78–180	ng/g w.w.	Takahashi, 1999
Other primary consumers	13	<9.0–25	5.4–34	12–45	26–104	ng/g w.w.	Takahashi, 1999
Diverse fish species, whole body	10	<9.0–15	1.8–32	5.1–210	15–257	ng/g w.w.	Takahashi, 1999
Dall's porpoise, liver	3	50–120	180–600	110–310	340–1,000	ng/g w.w.	Tanabe, 1998

d.w., dry weight; w.w., wet weight.

Annex I–Table 18 - Ranges of Concentrations of NPEs in the Canadian Environment (Total Number of Sites, Total Number of Samples)

Environmental Compartment	Site Type	4-NP	NP1EO	NP2EO	NP3–17EO	NP1EC	NP2EC
Effluents (µg/liter)	Textiles						
	Untreated	2.68–13.33 (2, 5)	37.17–257.09 (2, 5)	106.31–591.98 (2, 5)	798.42–8811.24 (2, 5)	<0.45 (1, 2)	<0.45 (1, 2)
	On-site secondary treatment	0.09–3.56 (2, 4)	1.12–4.10 (1, 2)	0.93–3.92 (1, 2)	2.07–315.45 (2, 3)	0.74–5.2 (2, 4)	<0.45–55.13 (2, 4)
	Going to MWWTP	0.23–25.62 (9, 14)	0.74–69.15 (10, 14)	0.64–284.51 (10, 14)	50.18–5767.65 (10, 14)	<0.45–1.90 (5, 7)	<0.45–2.80 (5, 7)
	Pulp and paper						
	Prior to 1998	<0.02–26.20 (14, 33)	<0.02–3780.00 (13, 32)	<0.02–67.84 (14, 33)	—	—	—
	After 1998	<0.10–4.3 (19, 19)	<0.10–6.90 (3, 3)	<0.10–35.60 (3, 3)	5.90–28.80 (3, 3)	<1.00–10.13 (15, 15)	<1.00–32.32 (15, 15)
	MWWTP						
	Primary	<0.02–62.08 (8, 21)	0.07–56.13 (10, 26)	0.34–36.33 (10, 26)	4.81–735.20 (8, 22)	1.17–11.00 (3, 7)	1.01–5.20 (3, 7)
	Secondary	0.12–4.79 (21, 54)	<0.02–43.37 (20, 46)	<0.02–32.62 (20, 46)	1.00–52.82 (16, 36)	2.15–74.97 (14, 34)	2.15–45.40 (14, 34)
	Tertiary	<0.02–3.20 (7, 37)	0.30–26.4 (7, 37)	0.25–12.45 (7, 37)	0.40–18.00 (6, 35)	2.15–48.58 (6, 34)	2.15–59.46 (6, 34)
	Lagoon	0.75–2.15 (5, 5)	0.34–0.90 (5, 5)	0.03–0.90 (5, 5)	1.00–2.10 (4, 4)	2.15–2.6 (4, 4)	2.15–3.00 (4, 4)
Aquatic (µg/liter)	Rivers	<0.02–4.25 (25, 90)	<0.02–2.30 (12, 51)	<0.02–2.45 (12, 51)	0.11–17.56 (3, 27)	0.44–3.17 (1, 37)	0.81–4.30 (1, 37)
	Lakes	<0.02–0.06 (5, 5)	<0.02–5.07 (4, 4)	<0.02 (4, 4)	—	—	—
	Harbors	<0.02–0.98 (12, 31)	<0.02–10.29 (12, 26)	<0.02–10.43 (12, 26)	—	—	—
Benthic (µg/g)		<0.02–72.20 (23, 58)	<0.02–38.12 (6, 14)	<0.02–6.02 (6, 14)	0.02–0.17 (1, 4)	—	—
Soil/sludge (µg/g)		0.74–1260 (30, 107)	2.90–1825.29 (28, 90)	1.52–297.21 (28, 90)	0.43–215 (28, 90)	<0.30–8.70 (17, 66)	<0.30–26.0 (17, 66)

MWWTP, municipal waste water treatment plant. Source: Servos (1999).

Annex I–Table 19 - Comparison of PCDD, PCDF and PCB Concentrations (pg/g lipid weight) in Humans

	Sweden, breast milk, 1997[a]	Norway, males 40–54, blood, median[b]	New Zealand, both genders, 35–49, serum, 1997, mean[c]	Arkansas, USA, 1991, mean[d]	Canada, blood donors, 1994, mean[e]
N	20	10	12	70	30
PCDDs					
2,3,7,8-TCDD	2	3.1	2.1	2.8	2.2
1,2,3,7,8-PeCDD	4	5.6	4.9	6.6	7.6
1,2,3,4,7,8-HxCDD	ND	2.2	2.8	9.0	
1,2,3,6,7,8-HxCDD	21	14.2	20.1	70.8	69.1[f]
1,2,3,7,8,9-HxCDD	ND	3.8	4.0	9.4	
1,2,3,4,6,7,8-HpCDD	30	34.7	38.2	124	NR
OCDD	100	470	399	971	NR
Sum of PCDDs	157	532	477	1194	NR
TEQ (PCDDs)	8.4	11.2	10.1	19.6	18.7
PCDFs					
2,3,7,8-TCDF	ND	2.9	< 0.7	3.1	NR
1,2,3,7,8-PeCDF	ND	1.6	< 0.5	1.6	NR
2,3,4,7,8-PeCDF	11	15.5	3.8	5.5	8.0
1,2,3,4,7,8-HxCDF	4	7.4	2.2	8.0	15.1[f]
1,2,3,6,7,8-HxCDF	3	7.7	2.6	5.3	
2,3,4,6,7,8-HxCDF	ND	3.8	0.8	3.8	
1,2,3,7,8,9-HxCDF	ND	0.9	< 0.4	1.8	
1,2,3,4,6,7,8-HpCDF	5	13.3	9.7	21.3	
1,2,3,4,7,8,9-HpCDF	< 1	1.1	1.1	NA	NR
OCDF	< 4	6.1	20.3	6.9	NR
Sum of PCDFs	23	60.2	40.5	57.3	NR
TEQ (PCDFs)	6.3	10.3	2.5	4.2	5.5
Sum of PCDDs and PCDFs	**14.7**	**21.5**	**12.6**	**23.8**	**24.2**
Non-*ortho*-PCBs					
CB-77	16	NA		12.6	ND
CB-81	-	NA		8.5	NR
CB-126	76	100.7		18.4	60
CB-169	39	72.7		17.9	43
Sum of non-*ortho*-PCBs	131	173.4		57.4	103
TEQ (non-*ortho*-PCBs)	8.0	10.8		2.0	6.4
Mono-*ortho*-PCBs					
CB 105	4,000	9,200			9,800
CB 114	ND	ND			NR
CB 118	13,000	32,200			19,000
CB 123	NA				ND
CB 156	6,000	26,200			8,600
CB 157	2,000	4,400			ND
CB 167	ND				ND
CB 189					ND
Sum of mono-*ortho*-PCBs	25,000	72,000			37,400
TEQ (mono-*ortho*-PCBs)	5.7	19.4			7.2
Total TEQ	**28.4**	**51.7**			**37.8**

[a]Norén and Meironyté, 2000. [b]Johansen et al., 1996. [c]Bates et al., 1999. [d]Anderson et al., 1998. [e]DeWailley et al., 1996. [f]Reported as the sum of the hexas.

Annex I–Table 20 - Maternal Plasma Concentrations of PCBs (geometric means, µg/kg lipid): Circumpolar Study 1994–1996 (AMAP)

	Canada (*n* = 67)	Greenland (*n* = 117)	Sweden (*n* = 40)	Norway (*n* = 60)	Iceland (*n* = 40)	Russia (*n* = 51)
PCBs (as Aroclor 1260)	439	1,577	606	458	590	570
CB 28	1.4	2.6	2.5	2.9	4.1	3.4
CB 52	1.7	3.8	2.0	1.8	2.2	2.3
CB 99	11.5	29.1	6.4	6.7	9.5	20.8
CB 101	1.8	4.3	1.5	1.4	1.9	2.4
CB 105	2.2	7.3	1.9	2.2	3.7	8.2
CB 118	8.8	33.7	11.4	10.5	16.2	31.3
CB 128	1.1	1.5	1.0	1.3	1.3	1.5
CB 138	29.6	118	47.4	35.1	45.7	49.8
CB 153	54.7	185	69.3	53.0	67.8	59.8
CB 156	5.0	15.4	8.6	6.3	8.0	9.0
CB 170	9.7	34.4	18.6	12.1	16.4	10.0
CB 180	26.6	82.5	34.1	25.3	34.4	20.5
CB 183	2.5	12.5	5.9	3.7	5.2	3.7
CB 187	10.2	41.3	11.0	10.3	13.3	8.1
14 PCB congeners	**167**	**571**	**222**	**173**	**230**	**231**

Annex I–Table 21 - Concentrations of PCDD (1987) and PCDF (1992–1993) Expressed as TEQ in Mothers' Milk from 18 Countries throughout the World

		1987/1988				1992/1993				
Country	Area	*n*	TCDD/F (pg TEQ/g)	*n*	Σ [6 PCB] (ng/g)	*n*	TCDD/F (pg TEQ/g)	no-PCB (pg TEQ/g)	[105+118] (pg TEQ/g)	Σ [6 PCB] (ng/g)
Albania	Tirana					10	4.8	1.3	1.1	63
Austria	Tulln	51	18.6			21	10.9	9.4	3.0	303
Belgium	Liege	21	40.2	21	609	20	27.1	1.7	3.1	306
Canada	Maritimes	19	15.6			20	10.8	2.9	1.2	86
	Quebec	34	18.1			20	13.4	5.1	1.7	137
	Ontario	76	17.6			20	18.1	5.8	1.8	128
	Pairies	31	19.4			20	14.6	2.3	0.9	58
	British Columbia	23	23.0			20	15.7	2.5	1.0	70
	Hudson Bay					5	20.9	13.3	8.0	1361
Croatia	Zagreb	41	11.8	41	450	13	13.5	5.2	2.7	219
Czech Republic	Kladno					11	12.1	2.5	3.5	532
Denmark	7 different cities	42	17.8	10	830	48	15.2	2.3	2.2	209
Finland	Kuopio	31	15.5	31	203	24	12.0	1.0	1.4	133
Germany	Berlin	40	32.0			10	16.5	9.0	2.7	375
Hungary	Budapest	100	9.1			20	8.5	0.8	0.8	61
Netherlands	20 different regions	10	34.2	96	272	83–104	23.5 (8.4–63.1)	8.8 (2.8–21.7)	2.9 (0.8–6.9)	273 (102–606)
Norway	Tromsö	11	18.9	10	562	10	10.1	16.1	3.4	273
Lithuania	Vilnius city					12	13.3	11.6	8.9	322
Pakistan	Lahore					14	3.9	1.9	0.4	19
Slovak Republic	Michalovce					10	15.1	6.4	7.0	1015
Spain	Bizkaia					19	19.4	6.7	3.9	461
Ukraine	Kiev nr.1					5	11.0	9.3	5.6	264
UK	Glasgow		29.1			23	15.2	2.6	1.3	131

Annex I–Table 22 - Comparison of PCDD/PCDF Levels by Age (New Zealand)

Congener	Total Population	Positives (n = 60)	15–24	25–34	35–49	50–64	>65
PCDDs							
2,3,7,8-TCDD	0.7–7.2	58	1.1	1.4	2.1	3.0	4.3
1,2,3,7,8-PeCDD	2.0–9.3	60	2.5	3.6	4.9	6.0	7.4
1,2,3,4,7,8-HxCDD	1.0–5.9	58	1.3	2.0	2.8	3.5	4.6
1,2,3,6,7,8-HxCDD	<0.7–44.8	58	8.7	15.6	20.1	25.4	36.2
1,2,3,7,8,9-HxCDD	1.7–8.3	59	2.6	3.4	4.0	4.8	6.4
1,2,3,4,6,7,8-HpCDD	14.2–85.9	58	21.0	32.3	38.2	48.9	57.2
OCDD	143–961	60	203	345	399	403	506
Sum of PCDDs			240	403	471.1	494.6	622.1
TEQ (PCDDs)							
PCDFs							
2,3,7,8-PCDF	<0.2–0.7	23	< 0.7	<0.4	<0.7	0.4	0.3
1,2,3,7,8-PeCDF	<0.2–0.7	12	< 0.7	<0.4	<0.5	0.3	< 0.3
2,3,4,7,8-PeCDF	1.8–8.3	60	2.4	2.9	3.8	5.1	6.1
1,2,3,4,7,8-HxCDF	0.9–4.3	60	1.5	1.7	2.2	2.9	3.3
1,2,3,6,7,8-HxCDF	1.1–4.6	60	1.6	2.2	2.6	3.2	3.8
2,3,4,6,7,8-HxCDF	0.3–1.5	56	0.5	0.7	0.8	1.0	1.0
1,2,3,7,8,9-HxCDF	<0.2–<2	0	<0.6	<0.4	< 0.4	<0.4	< 0.3
1,2,3,4,6,7,8-HpCDF	3.6–21.7	30	<20	<30	9.7	5.0	6.3
1,2,3,4,7,8,9-HpCDF	0.2–1.2	29	<1	0.5	1.1	<2	<0.7
OCDF	0.6–203	60	2.0	19.3	20.3	8.4	6.5
Sum of PCDFs			8	27.3	40.5	26.3	27.6
TEQ (PCDFs)							
Sum of PCDDs and PCDFs[a]			**249**	**443**	**499**	**533**	**649**
PCDD and PCDF TEQ			**6.6**	**9.4**	**12.4**	**16.1**	**21.4**

[a]Values from reference; not the same as adding the columns.

Annex I–Table 23 - Concentrations (ng/g lipid weight) of PBDEs in Human Milk and Blood

Gender	*N*	Sampling Year	Specimen	Geographical Area	Measure-ment	BDE47 (ng/g lipid weight)	BDE47 (range) (ng/g lipid weight)	PBDE	Reference (First author, year)
F	20	1990	Milk	Sweden	Pool	0.81	–	1.21	Meironyté, 1999
F	40	1997	Milk	Sweden	Pool	2.28		4.02	Meironyté, 1999
M	4	1994	Adipose	Sweden	Single		1.7–4.9	3.8–7.7	Meironyté, 2001
F	10	1992	Milk	Canada	Median	1.75	0.31–18.7	3.14	Ryan, 2000
					Mean	3.39		5.8	Ryan, 2000
F	200	1981/1982	Milk	Canada (wide)	Pool			0.2	Ryan, 2000
F	100	1992	Milk	Canada (wide)	Pool			16.2	Ryan, 2000
F	11	1994–1998	Milk	Finland	Median	0.77			Strandman, 2000
					Mean	1.1	0.36–2.80	2.2	Strandman, 2000
F	8	1985	Blood	Germany	Median	1.8		2.6	Schröter-Kermani, 2000
M	8	1985	Blood	Germany	Median	2.6		3.4	Schröter-Kermani, 2000
F	10	1999	Blood	Germany	Median	2.8		4.3	Schröter-Kermani, 2000
M	10	1999	Blood	Germany	Median	3.4		5.4	Schröter-Kermani, 2000
F	20	1997	Blood	Sweden	Median	1.6	<1–?	3.3	Sjödin, 1999
M	20	1992	Blood	Sweden	Median	0.4	0.1–2.5		Sjödin, 2000
M	19	1992	Blood	Latvia	Median	0.26	0.1–0.72		Sjödin, 2000
M	12	1992	Blood	Sweden	Median	2.2	0.96–5.7		Sjödin, 2000
M	26	1992	Blood	Latvia	Median	2.4	1.4–5.5		Sjödin, 2000
M + F	12 + 12	1998	Blood	Japan	Median	0.5	0.1–2.0	4.0	Nagayama, 2000
F	5	1995	Breast adipose	California, USA			7–28		She, 2000

F, female; M, male.

Chapter 7: Causal Criteria for Assessing Endocrine Disruptors—A Proposed Framework

7.1 Introduction

To create an objective and unbiased assessment of the hypothesis that chemicals with endocrine activity may be having adverse effects on laboratory animals, wildlife populations, and humans, all of the relevant information needs to be considered in an organized and structured manner. The challenge of this task stems from the vast number of studies conducted, the improbability that a single study could provide all the necessary information to link an exposure scenario to a particular health outcome in wildlife or humans, and the diverse circumstances (e.g., varied experimental conditions, numerous end points) from which data have been generated. Therefore, this chapter proposes the use of an organized framework, based on criteria modified by Bradford-Hill (1965), Fox (1991), and Ankley et al. (1997), to be used in the assessment of relationships between exposures to potential EDCs and altered health outcomes. It is important to recognize that the goal of this approach is not to provide point estimates of the association, as is case for quantitative meta-analyses (Greenland, 1998), but to reconcile different results from different studies as in done in qualitative meta-analyses (Cook et al., 1994). Examples of studies are included to illustrate how this framework can be used to assess causal relationships between "exposure and effect" and whether these associations involved endocrine-mediated events (see Tables 7.1 and 7.2). These examples are described in greater detail in other chapters of this assessment.

This structured, framework approach acknowledges that 1) there are a number of scientific uncertainties, 2) a degree of scientific judgment is involved, and 3) assessments are likely to change as additional information becomes available. This approach can identify key data gaps and research needs (see Chapter 8) that may reduce the uncertainties associated with the study of EDCs. Also, it should be noted that these assessments are qualitative determinations of the current overall state of the science. They are not quantitative risk assessments that relate specific exposure situations to probabilities of adverse effects. The objective of the framework is to provide a tool to evaluate the myriad of divergent and, at times, discordant data sets. In doing so, key research gaps may be highlighted and more informed assessments might be promoted in the future. By necessity, this approach is most useful when applied to the examination of a large body of evidence in the search for cause-and-effect relationships. It is less useful for identifying new signals in the environment that could be suggestive of endocrine disruption, although the establishment of the framework should still serve to guide research that could build the case for causality by pinpointing the key gaps in our knowledge.

List of Abbreviations

AhR Arylhydrocarbon receptor
DDE Dichlorodiphenyl dichloroethylene
DDT Dichlorodiphenyl trichloroethane
EDCs Endocrine-disrupting chemicals
GLEMEDS Great Lakes embryo mortality, edema, and deformity syndrome
Ig Immunoglobulin
PCBs Polychlorinated biphenyls
PCDDs Polychlorinated dibenzodioxins
PCDFs Polychlorinated dibenzofurans
T_4 Thyroxine
TBT Tributyl tin
TCDD 2,3,7,8-Tetrachlorodibenzyl-*p*-dioxin
TSH Thyroid-stimulating hormone
USA United States of America

7.2 Elements of the Proposed Framework

The framework begins with a clear statement of the *hypothesis* under examination, which contains two distinct elements. First, the outcome of concern (e.g., a specific human disease or status of an ecological species) is linked to a putative stressor that is acting on the individual or population. Second, exposure to the stressor results in endocrine-mediated events that ultimately result in the outcome of concern. These elements need to be clearly stated in order to evaluate the scientific evidence regarding their potential relationship. The evaluation of the scientific evidence utilizes five aspects: 1) temporality, 2) strength of the association, 3) consistency of the observations, 4) biological plausibility of the effect, and 5) evidence for recovery following diminution of the stressor. The aspect of specificity of the association, a traditional component of causality in the epidemiological setting, is not included in this framework because some of the examined outcomes (e.g., semen quality) are quite apical in nature and influenced by many factors, and the component of biological plausibility covers the linkage between the mechanism of action and the outcome (e.g., estrogen mimics and vitellogenin induction in fish) and hence deals implicitly with specificity.

1) The aspect of *temporality* explores whether the presumed cause of the outcome of concern preceded the appearance of altered physiological states, rates of disease, or population health. Although information regarding the onset of exposure is often lacking, a few examples are included in which the temporal pattern of exposure precedes the observed effect.

2) The aspect of *strength of the association* examines a) the incidence rate of the outcome in a population, b) the extent to which other known risk factors may have contributed to this incidence, c) the risk that could be attributed to the exposure of concern, and d) the shape of the dose–response curve as determined either from laboratory or population-based studies.

3) The aspect of *consistency of the observations* examines how frequently similar or dissimilar conclusions are reached in the literature and discusses any apparent discrepancies. It also evaluates whether results came from multiple geographical areas, whether multiple species would be expected to react in a similar fashion, and whether studies employed similar dosages.

4) The aspect of *biological plausibility* examines multiple areas of research (e.g., basic aspects of biology, embryology, endocrinology, population dynamics, chemical/physical properties, etc.) that help determine the mechanism of action for the compounds of concern. Consideration of a substance's mechanism is critical because this criterion is central to the overall assessment of whether or not a substance is deemed to be an "endocrine disruptor." In this assessment, a substance meets the operational definition of an endocrine disruptor if it "alters the function of the endocrine system and consequently causes an adverse health effect in an intact organism, or its progeny, or (sub)populations."

5) The aspect of *evidence of recovery* examines whether the occurrence of the adverse outcome is reversible upon diminishment or cessation of the suspected exposure. When examining the issue of recovery, it is important to note that some effects may be developmentally imprinted, and hence recovery may only occur in

Table 7.1 - Illustrative Examples

Statement of Hypothesis		Evaluation Factor					Overall Strength of Evidence	
Outcome	Stressor	Strength of Temporality	Association	Biological Consistency	Plausibility	Recovery	For Hypothesis	For EDC Mechanism
Endometriosis in humans	TCDD, PCBs	ND	*	*	*	ND	Weak	Moderate
Impaired neurobehavioral development in humans	PCBs	****	***	***	***	ND	Moderate	Moderate
Perturbed immune function in humans	PCBs, TCDD	***	****	**	**	*	Moderate	Weak
Incidence of breast cancer in humans	DDT, DDE, and PCBs	*	*	*	**	ND	Weak	Weak
Imposex in marine gastropods	TBT	****	****	****	***	****	Strong	Strong
Decreased reproductive function in Baltic seals	PCBs	***	**	***	***	****	Strong	Moderate
GLEMEDS in birds	Polychlorinated halogens (PCBs)	****	****	****	****	****	Strong	Weak
Egg shell thinning in colonial waterbirds	DDE and other DDT metabolites	****	****	****	***	****	Strong	Moderate
Reproductive abnormalities in Lake Apopka alligators	Dicofol and agricultural pesticides	****	***	***	***	**	Moderate	Moderate
Developmental abnormalities and reproductive failure in Lake Ontario lake trout	Dioxins and coplanar PCBs	****	****	***	****	****	Strong	Weak
Vitellogenin induction in fish exposed to sewage treatment plant effluents in England	Estrogenic contaminants	****	****	***	****	**	Strong	Strong
Reproductive alterations in fish exposed to bleached Kraft mill effluent in Ontario	Bleached Kraft mill effluent	****	****	***	****	***	Strong	Strong

ND, no relevant data. This table summarizes the overall strength of evidence for each criterion of the framework (evaluation factors) developed to assess the potential effects of EDCs. Each criterion has been ranked from weak (*) to strong (****), and each element of the hypothesis (outcome, stressor, and EDC mechanism) is evaluated as either weak, moderate, or strong.

Table 7.2 Illustrative Examples (Status and Trend Data Only)

Statement of Hypothesis		Evaluation Factor					Overall Strength of Evidence		
Outcome	Stressor	Temporality	Strength of Association	Consistency	Biological Plausibility	Recovery	For Outcome	For Hypothesis	For EDC Mechanism
Reduction in semen quality and testis function in humans	Estrogenic and antiandrogenic chemicals	ND	ND for association * for effect	ND for exposure * for effect	***	ND	Weak	ND	Weak
Limb malformations in North American frogs	Unknown chemical etiology	ND	ND for association ** for effect	ND for exposure * for effect	**	ND	Strong	Weak	Weak

ND, no relevant data. This table summarizes the overall strength of evidence for each criterion of the framework (evaluation factors) developed to assess the potential effects of EDCs. Each criterion has been ranked from weak (*) to strong (****), and each element of the hypothesis (outcome, stressor, and EDC mechanism) is evaluated as either weak, moderate, or strong.

subsequent generations, or may even express themselves in subsequent generations that have not in themselves been exposed to the stressor.

7.3 Overall Strength of Evidence

The final part of the framework, *overall strength of evidence*, makes an evaluation regarding the relationship between an outcome of concern and exposure to a substance and whether or not these associations involve endocrine-mediated mechanisms. These concluding remarks are drawn from the five criteria described above.

7.4 Illustrative Examples—Status and Trends Observations

7.4.1 Semen Quality and Testis Function in Humans

Hypothesis: Global reductions in human semen quality over time are related to increasing exposure to estrogenic, antiandrogenic (identity unknown), or other as yet unidentified chemicals, during critical phases of testicular development.

Temporality: A number of studies from different parts of the world have shown significant declines in sperm count and semen volume in men over time. However, none of the studies of semen quality have included prenatal, childhood, or adult exposure assessments for estrogenic or antiandrogenic chemicals, and the decline began before the use of industrial chemicals became widespread, especially if one considers the effect to be due to exposures during fetal or early postnatal life.

Strength of association: There are no human data that directly address the proposed cause-and-effect relationship. Concerning strength of effect, the meta-analysis showed a decline in semen quality of around 50% over 50 years, or 1.5% per year for the USA and 3.5% per year for Europe.

Consistency: There are no data relating to consistency of effect with exposure to chemicals of concern. A meta-analysis of studies published between 1938 and 1990 from 20 countries showed declines in sperm count and semen volume in men over time. Of the subsequent longitudinal studies in single centers, 10 are consistent with a decline, 6 are consistent with improvement, and 8 show

no change in semen quality over time. The many potential confounders in semen quality studies (e.g., differing population characteristics, methods of semen collection and analysis) could explain the inconsistencies. Two "time to pregnancy" (fecundity) studies have also produced results that are not consistent with decreased semen quality (i.e., they have not observed a decline in fertility among couples), although it should be noted that many factors influence fertility.

Biological plausibility: Endogenous estrogens control testis development. However, prenatal exposure to pharmacological estrogens, including diethylstilbestrol, in humans is not associated with effects on fertility. Support for biological plausibility comes from human data for incidence trends in developmentally related end points (i.e., testicular cancer and male reproductive tract abnormalities). Further supportive evidence comes from experimental animal data showing adverse effects on male reproductive tract development and adult testicular function with exposure to estrogenic and antiandrogenic chemicals (e.g., estradiol, nonylphenol, methoxychlor, vinclozolin, phthalates, TCDD). The prenatal and perinatal periods are particularly vulnerable to disturbances of male reproductive tract development by these chemicals, whereas higher doses are required to affect testis function in the case of adult exposure.

Recovery: No relevant data.

Overall strength of evidence

For outcome, the evidence is judged to be weak. A global trend for declining semen quality is not supported by current data. Some studies show declines in certain regions or cities, whereas others have not found a decline, suggesting there may be regional trends but not a global trend. There is no evidence relating to the strength of the hypothesis because of the lack of exposure data.

There are no human data to support an EDC-related mechanism. However, the biological plausibility of the hypothesis remains strong, based on information from clinical experience and experimental systems.

7.4.2 Limb Malformations in North American Frogs

Hypothesis: Exposure to chemicals that influence endocrine function contributes to the increased prevalence of limb malformations in North American frog populations

Temporality: There has been a recent increase in the number of observations of deformed frogs captured over wide geographic regions of North America, suggesting that the incidence of such effects is increasing. The temporality is difficult to reconcile in the context of a general global reduction in chemical exposure.

Strength of association: The strength of association with chemical exposure is weak.

Consistency: In the absence of information on the stressors responsible for malformations, it is difficult to assess whether response are consistent across time or at different sites.

Biological plausibility: Current knowledge of the involvement of endocrine processes in the development of amphibians provides the underlying mechanistic basis that the effects seen in frogs may be a consequence of exposure to chemicals found in the environment. However, cause-and-effect relationships must be further examined.

Recovery: There are no data available.

Overall strength of evidence

For the outcome of concern, there is considerable evidence that the incidences of malformations in frog populations are high and/or rising. At this time, the evidence that there is a chemical etiology mediating these malformations or that this involves effects on endocrine function is weak.

7.5 Illustrative Examples—Nonstatus and Trend-Type Observations

7.5.1 Endometriosis in Humans

Hypothesis: Endometriosis in women is related to endocrine disruption mediated by exposure to TCDD and/or PCBs.

Temporality: Not assessable. Endometriosis is a common disease in women, but there are few data on temporal trends. Exposure to dioxins and PCBs is ubiquitous.

Strength of association: There are no estimates of the proportion of endometriosis attributable to TCDD/PCBs. The only case–control study gave an odds ratio of 7.6 (95% confidence interval, 0.87–169.7).

Consistency: Two studies reported an association between serum levels of TCDD and endometriosis, one with no dose response. One study reported an association between PCB exposure and endometriosis. Another study reported no association between endometriosis and serum PCBs or dioxins. No association was found in Seveso women exposed to high levels of TCDD.

Biological plausibility: There is a clear dose–response relationship between endometriosis and endogenous estrogens and exposure to pharmacological estrogens, but TCDD can oppose the effects of estrogen. Conflicting evidence comes from studies of endometriosis in monkeys; one positive for TCDD exposure, one showing a bimodal dose response for TCDD, and one negative for PCB exposure. In mice (but not rats), surgical induction of endometriosis is enhanced by exposure to relatively high doses of TCDD or 4-chlorodiphenyl.

Recovery: No relevant data.

Overall strength of evidence

Relative to the hypothesis of an association between a stressor and an outcome, evidence is judged to be weak because of conflicting data from humans and animals, lack of association in women exposed to high amounts of TCDD, and antiestrogenic effects of TCDD.

In humans, occurrence of endometriosis shows dependency on estrogen–progesterone balance, suggesting that an EDC-related mechanism may be possible.

7.5.2 Impaired Neurobehavioral Development in Humans

Hypothesis: Impaired neurobehavioral development in children is related to endocrine disruption mediated by exposure to PCBs.

Temporality: Impaired neurobehavioral development has been observed in association with prenatal and possibly early postnatal exposure to PCBs.

Strength of association: A range of adverse, persistent effects have been observed in offspring of mothers exposed to relatively high levels of PCBs (Yusho and Yu-Chen poisoning incidents). More subtle, less persistent effects have been observed in certain populations exposed to lower PCB levels.

Consistency: Although there are several studies reporting neurological effects in children exposed to low levels of PCBs, the outcomes measured in studies have showed some variability. In reviewing the evidence, consideration was given to the complexity in measuring neurological function in children, the different populations in which effects were reported, and the fact that the studies were not designed to be replicates of one another. In addition, the timing of exposure may be critical but has not yet been characterized, particularly with respect to postnatal exposure.

Biological plausibility: PCBs and other agonists of the AhR are known to interfere with thyroid hormones and sex hormone action,

which are known to be critical for normal brain development. PCBs are known to have hypothyroid actions at exposure levels in certain populations. Evidence from experimental animal studies of neurobehavioral effects and elevated brain thyroxine deiodinase levels in offspring exposed to PCBs during gestation also supports biological plausibility.

Recovery: No relevant data.

Overall strength of evidence

Relative to the hypothesis of an association between a stressor and an outcome the evidence is judged to be moderate. Several human studies show a range (severe to minor) of adverse effects but within the same continuum of motor and mental developmental delays or impairments. There is appropriate temporality in relation to PCB exposure, a broad dose response across studies (but not necessarily within studies), and reasonable consistency, except with respect to the possible contribution of postnatal exposure. Interpretation is complicated by incomplete exposure measures in some studies (maternal serum and/or cord serum and/or milk).

There is limited direct human evidence of an EDC-related mechanism, with maternal serum/milk PCB/PCDD/PCDF/TCDD levels reported to be negatively associated with infant T_4 levels and positively associated with infant TSH levels, but T_4 and TSH still within their normal clinical range. Overall, the evidence is judged moderate.

7.5.3 Perturbed Immune Function in Humans

Hypothesis: Perturbations in immune function via changes in endocrine function are mediated by exposures to PCBs and TCDD.

Temporality: Most human data are derived from studies of *in utero* and/or accidental exposure. The outcomes were assessed after the exposure had occurred. However, no data are available regarding the presence of symptoms and/or baseline measurement of immune function cells prior to exposure.

Strength of Association: In children, *in utero* exposure to PCBs and TCDD is associated with abnormal measures of immune function cells and serum antibodies. In children exposed during the pre- and postnatal period, a higher prevalence of respiratory symptoms and other infectious diseases (but a lower prevalence of allergic disease) was observed. These observations span a range of exposure from very high (Yu-Cheng cohort) to background levels (Dutch breastfeeding study). In adults, alterations of immune function are observed in all but one study following both accidental and occupational exposure; no data are available for general environmental levels of exposure.

Consistency: At high levels of exposure, there is ample evidence for perturbed immune function in both children and adults. At lower levels, there is only one study in children and no data in adults.

Biological plausibility: *In vitro* and *in vivo* data suggest that TCDD induces AhR-mediated thymic atrophy. TCDD may also deplete thymocytes through either apoptosis or direct action on the bone marrow. These data qualitatively support the human studies.

Recovery: Longitudinal data from the Yusho cohort indicate decreased levels of serum IgA and IgM 2 years after exposure ceased, although an increased prevalence of respiratory symptoms persisted for longer times.

Overall Strength of Evidence

Relative to the hypothesis of an association between a stressor and an outcome, the evidence is deemed moderate. Most studies in children and adults indicate moderate associations between high levels of exposure to TCDD and measures of immune function. Evidence for perturbed immune function following exposure to lower levels of PCBs is limited to one study, and more evidence is clearly needed.

Regarding an EDC-related mode of action, the overall assessment is weak. Both *in vivo* and *in vitro* data suggest that TCDD perturbs the activity of the thymic epithelium possibly due to AhR-mediated thymic atrophy.

7.5.4 Incidence of Breast Cancer in Humans

Hypothesis: Increased incidences of breast cancer are caused by exposure to organochlorine chemicals (e.g., PCBs, DDT, and metabolites) possessing estrogenic activity.

Temporality: Little information is available on the patterns of organochlorine exposure from birth to menopause in case–control studies. Because organochlorines are biologically persistent, the current exposure measures may represent past exposure. The timing and magnitude of the exposure during the full life span may be critical in order to detect effects on breast cancer risk.

Strength of Association: Most studies show no association between breast cancer and organochlorine exposures; the few positive studies are weak.

Consistency: For DDT and its metabolites, there are seven reported positive associations that are statistically significant out of 34 studies in total. For PCBs there are five reported positive associations that are statistically significant out of 24 studies in total.

Biological plausibility: There is strong evidence that physiological levels of naturally occurring estrogens can contribute to breast cancer risks in women and that the cumulative lifetime exposure to endogenous estrogens correlates with the incidence of breast cancer in populations. Additional exposure to estrogenic chemicals could therefore plausibly increase the risk of disease. However, the potency of "organochlorine" estrogens is low compared with endogenous hormones and phytoestrogens, and therefore, any added risks from PCB or DDT exposure would be very small relative to the contributions of endogenous estrogens and probably not detectable in case–control studies.

Recovery: No relevant data

Overall Strength of Evidence

For breast cancer as an outcome, the evidence that the incidences are increasing is moderate based on several valid and well-conducted surveys. Breast cancer screening practices and early detection may have contributed to the reported increases.

In terms of stressors, the evidence is weak in support of the hypothesis that exposure to PCBs, DDT, and other organochlorines contribute to increased risk based on the lack of consistency of the results, weak associations, and questions of biological plausibility. The evidence is also weak for an EDC mode of action.

7.5.5 Imposex in Marine Gastropods

Hypothesis: TBT originating from antifouling paints used to treat boat hulls induces a form of pseudohermaphroditism (termed imposex) in female gastropods by an endocrine-disrupting mechanism.

Temporality: The use of TBT has been associated with increased incidence of imposex and population declines.

Strength of association: The frequency of imposex and the degree of penis development in females are related to the degree of TBT exposure. Laboratory studies have confirmed the effects of TBT on imposex in neogastropod mollusks. A related condition termed intersex, which involves TBT-induced changes in the oviduct, occurs in littrinid gastropods.

Consistency: There is strong evidence of worldwide effects on gastropod populations. Imposex is a generalized response in marine gastropods exposed to TBT, because effects have been seen in over 100 species in almost 50 genera.

Biological plausibility: The effects of TBT have been reproduced in controlled laboratory studies. Current understanding of the mechanism of action of TBT is incomplete, although it appears to have an endocrine basis related to mechanisms that contribute to the elevation of androgen levels (inhibition of aromatase).

Recovery: Banning the use of TBT in antifouling paints has been effective in reducing environmental concentrations of TBT and a corresponding decrease in the incidence of imposex and/or increased reproductive success of previously affected gastropod populations.

Overall strength of evidence

Relative to the hypothesis of an association between a stressor and an outcome, the evidence that TBT affects sexual development and reproduction in female gastropods represents one of the strongest case studies showing that exposure to environmental chemicals contributes to population-level impacts.

For an EDC-related mode of action, the evidence is considered strong in that alterations in aromatase activity have been implicated in the alteration of steroid hormone profiles (elevated androgen levels).

7.5.6 Decreased Reproductive Function in Baltic Seals

Hypothesis: Exposure to persistent organochlorine contaminants, including PCBs derivatives, contributes to reproductive toxicity in Baltic seals through an endocrine dependent mechanism.

Temporality: High levels of persistent organochlorine contaminants are strongly correlated with reduced reproductive success of Baltic seal populations. Exposures to organochlorine contaminants are consistent with the induction of a range of abnormalities including uterine alterations that are thought to contribute to sterility and lowered reproductive success.

Strength of association: In general, the link between adverse reproductive outcomes and chemical etiology is weak. This is further complicated by the presence of a range of pathological lesions and immune function alterations where the linkages with reproductive outcome is unknown.

Consistency: It has been difficult to evaluate the consistency of responses in the field setting when so many factors are variable. Nevertheless, observations in marine mammals on a global scale provide evidence that Baltic seals may be susceptible to alterations in reproduction.

Biological plausibility: The results of semi-field studies with Baltic seals and harbor seals provide general links with organochlorine exposure and reproductive outcome, but there are complications in establishing cause-and-effect relationships because of inadequate study designs.

Recovery: There is little opportunity to examine the consistency of the response and recovery over time. In general, reproduction has been improving as the levels of contaminants decline.

Overall strength of evidence

Relative to the outcome of concern, there is considerable evidence that the reproductive success of Baltic seal populations has been impacted and that these exposures have altered adrenal gland function in members of the exposed population. However, because the link between altered adrenal function and reproductive impairment has not been clearly established, the overall evidence pertaining to the existence of an EDC-related mode of action is moderate.

7.5.7 GLEMEDS

Hypothesis: Developmental abnormalities and embryo mortality in colonial fish-eating water birds in the Great Lakes of the USA are a consequence of exposure to persistent organochlorine compounds including PCBs, which act though an endocrine-dependent mechanism.

Temporality: Several fish-eating bird species (e.g., double-crested cormorants, herring gulls, Forster's and common terns, bald eagles) in the Great Lakes experienced severe population declines from the 1940s to early 1970s. This was associated with exposure to high levels of persistent organochlorine compounds. Incidences of GLEMEDS have decreased as levels of α compounds in the environment have decreased.

Strength of association: Ecoepidemiological studies point to the syndrome being mediated by exposure to persistent organochlorine compounds, particularly PCBs, which bioaccumulate in the egg. Laboratory experiments have been instrumental in establishing the relationship between exposure to AhR agonists and the GLEMEDS syndrome.

Consistency: The similar pattern of response seen in several avian species at different sites within the Great Lakes adds to the weight of evidence linking exposure and outcome. Similar responses were observed in chickens exposed to PCDFs and PCDDs.

Biological plausibility: Several studies confirm that early life stages are particularly sensitive to chemicals that act through the AhR.

Recovery: There have been reductions in the extent and severity of symptoms of GLEMEDS with reductions in environmental exposures of organochlorine compounds.

Overall strength of evidence

The evidence for a causal relationship between the postulated stressor and the GLEMEDS outcome is strong (as demonstrated in several areas, with supported evidence from laboratory experiments).

Because the outcome (embryonic edema and mortality) is not necessarily related to alterations in endocrine function, and because of the lack of mechanistic studies in model systems, there is considerably less certainty that an EDC mode of action is involved in this observation.

7.5.8 Eggshell Thinning in Colonial Waterbirds

Hypothesis: Eggshell thinning caused by exposure to DDE results in cracked or broken eggs and other adverse reproductive effects through an endocrine-mediated mechanism.

Temporality: During periods of high use of DDT as an insecticide in North America and Europe, DDE-induced eggshell thinning nearly resulted in the extinction of several avian species.

Strength of association: The strength of association between DDE exposure and effects on eggshell thickness is strong, with similar effects being seen in both North American and European studies.

Consistency: Adverse responses to DDE have been observed in a range of species at multiple sites. The sensitivity to DDE-induced eggshell thinning varies markedly among avian species, and different mechanisms may contribute to the types of eggshell defects seen in different species.

Biological plausibility: Numerous laboratory-based and *in vitro* studies confirm the relationship between DDE exposure and adverse reproductive outcome; however, the laboratory studies have yielded somewhat inconsistent results regarding the mode of action.

Recovery: Many species that are sensitive to eggshell thinning have experienced dramatic population increases as a result of reduced exposure to DDT and its metabolites.

Overall strength of evidence

Relative to the outcome of concern, there is strong evidence that eggshell thinning results from exposure to DDE.

There continues to be uncertainty with respect to the precise mechanism of action of DDE and the extent to which this involves alterations in endocrine function. The likelihood that the outcome involved an EDC mode of action is considered moderate, due primarily to the relationship with altered prostaglandin synthesis in the mucosa gland of sensitive species.

7.5.9 Reproductive Abnormalities in Lake Apopka Alligators

Hypothesis: Reproductive tract and endocrine abnormalities observed in Lake Apopka alligators are a result of exposure to chemicals originating from a spill of the pesticide dicofol (including its metabolic or environmental breakdown products) or ongoing agricultural practices.

Temporality: Reproductive failure leading to reductions in the numbers of neonate and juvenile alligators, developmental abnormalities of the reproductive tract and the male phallus, and abnormal sex steroid levels were observed in Lake Apopka alligators in the years following a chemical spill. These response patterns have persisted for over 15 years following the chemical spill.

Strength of association: This is rated as strong because similar response patterns have persisted over time.

Consistency: There is a high degree of consistency in responses seen within Lake Apopka (in general, the response patterns observed in other locations are highly variable).

Biological plausibility: It is evident that alligators in Lake Apopka have been exposed to chemicals that are known to interact with endocrine receptors or contribute to reproductive toxicity. However, data on cause-and-effect relationships and laboratory-based exposure–response studies are limited.

Recovery: Although there has been some indication of a reduction in the severity of developmental abnormalities and a gradient of responses has been observed within Lake Apopka, there are limited data available with respect to recovery.

Overall strength of evidence

Regarding the outcome, there is a relatively strong indication of adverse effects of chemical exposure on the alligator population in Lake Apopka.

Although there are supporting laboratory data regarding particular chemical stressors and altered endocrine status in the alligator embryo, the current understanding of whether an EDC-related mode of action is responsible is weak.

7.5.10 Vitellogenin Induction in Fish Exposed to Sewage Treatment Plant Effluents in England

Hypothesis: Estrogenic compounds in the effluents from sewage treatment plants throughout England contribute to increases in vitellogenin production and intersex in fish living in the receiving environment.

Temporality: Both vitellogenin production in males and the incidence of intersex in fish are highest immediately downstream of sewage treatment discharges and generally decrease with distance downstream. Caging of naive fish in the vicinity of sewage effluent discharge provides evidence for the strength of association and outcome, at least in terms of vitellogenin production.

Strength of association: Both *in situ* exposure of caged fish and exposure of fish to sewage effluents in the laboratory provide confirmation that effects on vitellogenin production and gonad development are mediated by chemicals originating in the sewage effluent. Other studies at various locations throughout Europe and North America have confirmed the link between sewage treatment plan effluents and vitellogenin induction; far less is known regarding effects on gonadal development.

Consistency: This is rated high based on the temporal patterns of response and the consistency between different geographic areas.

Biological plausibility: Laboratory experiments have established that estrogenic compounds are responsible for increased vitellogenin production and contribute to effects on gonadal development that may lead to intersex. Chemical fractionation studies have confirmed that sewage effluents are significant sources of estrogens (synthetic estrogens and industrial chemicals with estrogenic activity) and that these are present in amounts that are likely to mediate the observed biological effects. There is variability in the concentration response to vitellogenin induction by estrogens in male fish, and species may respond differently to similar exposure levels.

Recovery: Little is known of the long-term effects following exposure to estrogenic compounds.

Overall strength of evidence

For the outcome of vitellogenin induction in male fish downstream of sewage treatment plants, several well-conducted studies convincingly demonstrate a causal relationship.

Relative to an EDC mode of action, environmental monitoring information, laboratory studies, and research on biological plausibility indicate a strong likelihood that an EDC mode of action is involved. The presence of estrogenic compounds in sewage treatment effluents represents one of the best examples illustrating the linkage between exposure to an EDC and an outcome.

7.5.11 Developmental Abnormalities and Reproductive Failure in Lake Ontario Lake Trout

Hypothesis: Exposure to TCDD and coplanar PCBs contributes to mortality during early development and reduced reproductive success in Lake Ontario lake trout through an endocrine-mediated mechanism.

Temporality: Lake trout populations in Lake Ontario declined precipitously during the period when environmental levels of persistent bioaccumulative organochlorine chemicals were highest.

Strength of association: Laboratory studies have shown that exposure to AhR agonists induce blue-sac disease, which was responsible for early-life-stage mortality in the embryos of artificially spawned lake trout. Subsequent retrospective studies (based on measured PCB, PCDF, and PCDD residues in dated sediment cores) have established a strong relationship with the observed historical trends in lake trout reproduction, including recent signs of successful reproduction. Collectively, these results confirm that AhR agonists are primary contributors to early-life-stage mortality and adverse population level impacts.

Consistency: In addition to limited field observations, laboratory studies have established a linkage between dioxin equivalent concentrations and outcome.

Biological plausibility: Laboratory-based studies confirmed that the early life stage is sensitive to AhR agonists, including TCDD and coplanar PCBs, and that the observed pathology (e.g., blue-sac disease) was consistent with observations in embryos from field-collected lake trout.

Recovery: Recent studies have shown that there are reductions in the incidence of blue-sac disease in lake trout, associated with a decline in dioxin equivalent concentrations in Lake Ontario.

Overall strength of evidence

Both field studies and laboratory research provide ample evidence that the outcome of early life stage mortality in lake trout is related to exposure to AhR agonists, including TCDD and PCBs.

Despite the strong association between the stressor and the outcome, experimental studies have yet to establish an EDC-related mode of action.

7.5.12 Reproductive Alterations in Fish Exposed to Bleached Kraft Pulp Mill Effluent in Ontario

Hypothesis: Chemicals present in the effluent from a bleached Kraft pulp mill at Terrace Bay, Ontario, contribute to endocrine dysfunction and delayed reproduction in white sucker fish in the surrounding environment.

Temporality: A series of studies have shown that white sucker fish exposed to the effluent from a bleached Kraft pulp mill at Terrace Bay, Ontario, exhibited changes in reproductive development, including delayed sexual maturity, reduced gonadal growth, and alterations in plasma sex steroid hormone levels. Studies with caged fish in effluent or with fish exposed to effluent in the lab provide evidence of a temporal link between exposure and changes in sex steroid levels.

Strength of association: Studies examining fish downstream of other pulp mills in Canada and Sweden support the hypothesis that chemicals in the effluent contribute to adverse reproductive responses. Fish exposed to effluent from pulp mills also exhibited adverse reproductive effects.

Consistency: This is rated high, because similar responses have been observed over a 10-year period (irrespective of changes in effluent treatment and bleaching technologies).

Biological plausibility: Bleached Kraft mill effluent is a complex mixture of chemicals that make it difficult to identify specific bioactive compounds. Evidence that fish living in these surroundings are exposed to endocrine-active chemicals comes from studies showing that they rapidly accumulate ligands for sex steroid receptors (androgen, estrogen, and sex steroid binding protein) and the AhR. Both lab and field studies provide evidence of adverse effects being linked to effluent exposure.

Recovery: Reproductive endocrine changes are diminished in fish collected during periods of reduced effluent discharge (e.g., mill shutdown). There is also a rapid decrease in concentrations of endocrine-active ligands in the fish following transfer to clean water.

Overall strength of evidence

For the outcome of altered reproductive outcome in fish living in the vicinity of a bleached Kraft mill, there is compelling evidence that chemicals in the effluent are responsible for changes in endocrine function and reproductive performance of fish.

Although the active compounds responsible for the biological effects have not been identified, the findings are consistent with an EDC mode of action. The evidence of altered steroid receptor function in exposed fish is deemed strong.

Chapter 8: General Conclusions and Research Needs

This assessment summarized the current state of the scientific knowledge regarding the potential effects of exposure to EDCs in humans and wildlife. Predominantly, the information was gathered from North American and European studies, which limits drawing conclusions on a worldwide basis. The potential risks to humans and wildlife posed by EDCs in many other areas of the world (particularly in developing countries) have not been addressed adequately to date. Although it is clear that certain environmental chemicals can interfere with normal hormonal processes, there is weak evidence that human health has been adversely affected by exposure to endocrine-active chemicals. However, there is sufficient evidence to conclude that adverse endocrine-mediated effects have occurred in some wildlife species. Laboratory studies support these conclusions.

Generally, studies examining EDC-induced effects in humans have yielded inconsistent and inconclusive results, which is responsible for the overall data being classified as "weak." This classification is not meant to downplay the potential effects of EDCs; rather, it highlights the need for more rigorous studies. This document has identified a number of inherent challenges and confounding factors that contribute to the difficulties in understanding the risks that EDCs pose to human health. The only evidence showing that humans are susceptible to EDCs is currently provided by studies of high exposure levels. Our understanding of the effects of chronic, low levels of EDCs are much more obscure. In particular, the relationship between early-life exposures to EDCs in humans and functioning in adult life is poorly understood. This is a concern because laboratory animal studies have indicated that early life stages may be especially sensitive to the effects of EDCs. Only recently have human epidemiological studies been conducted with the necessary rigor to sufficiently address potential cause-and-effect relationships in regards to EDC exposures.

Compared with humans, the evidence that wildlife have been affected adversely by exposures to EDCs is extensive. In part, this may reflect the fact that many studies on wildlife have been conducted in areas where it is known that the levels of environmental chemicals are high (e.g., point source discharges, the Great Lakes, the Baltic Sea area). These studies have focused predominantly on animals inhabiting aquatic ecosystems, which bioaccumulate certain EDCs and represent one of the largest sinks of environmental chemicals that may act as EDCs. Progress in establishing cause-and-effect relationships in wildlife has been aided by the ability to experiment with the species of concern under both laboratory and field conditions. Many of the challenges encountered in assessing the risks of EDCs to human health are also relevant to wildlife species. However, there are unique challenges to determining the potential effects of EDCs on wildlife compared with humans, including the large number of potential target species, varied life history strategies, differences in physiological mechanisms, and lack of basic understanding of endocrine regulation for many species.

List of Abbreviations

EDCs Endocrine-disrupting chemicals
NRC National Research Council

This assessment has clearly identified that there is little information on linkages between exposures to putative EDCs and health outcomes in both humans and wildlife. Progress has been made in the identification and quantification of a wide array of chemicals with endocrine-active properties. Predominantly, research efforts have focused on compounds that persist and bioaccumulate in organisms and their environment. Only recently have efforts been directed at exposure studies of less persistent compounds and in the development of biologically based assays, which would enable more direct assessments of endocrine-active compounds. Given the dynamic nature of the endocrine system, future efforts in the study of EDCs need more focus on the timing, frequency, and duration of exposure to these chemicals.

This assessment has clearly demonstrated that further research is necessary to address the uncertainties that remain in this field of study. Some specific research recommendations are cited in the preceding chapters of this document and have also been the subject of a number of international workshops (Kavlock et al., 1996; EC, 1996; Ankley et al., 1998; Kendall et al., 1998; NRC, 1999; Vos et al., 2000). Strengthening international collaborative efforts in the following broad research areas will help resolve uncertainties and should be considered of high priority:

1) **Biology underlying endocrine-mediated effects**
- Expand basic knowledge about endocrine systems in humans and wildlife.
- Elucidate the range of mechanisms by which endocrine disruption may interfere with reproductive/population success, immune function, neurobehavior, and development of cancer, at all levels of biological organization and at key stages of life cycles.

2) **Methodology**
- Develop improved methodologies for assessing dose–response relationships at environmentally relevant concentrations.
- Develop more specific and sensitive biomarkers for detecting endocrine-mediated effects in individuals and populations.

3) **Monitoring**
- Increase long-term monitoring of "sentinel" wildlife species to provide baseline data on population status.
- Improve international collaboration and cooperative research to assess the exposure and effects of EDCs on wildlife populations on a more global basis.
- Extend monitoring of trends in relevant human health outcomes to provide information that is comparable across regions and over time.

4) **Identifying endocrine disruptors**
- Continue to identify chemicals (persistent and nonpersistent, naturally occurring and anthropogenic) that are the most likely candidates for high-impact effects in populations at environmentally relevant concentrations.

- Identify "hot spots" for exposure or effects that warrant particular concern.
- Focus work on populations/subgroups most likely to be susceptible to endocrine disruptors.
- Assess the role of endocrine disruptors relative to other environmental stressors on the fitness of populations.

5) **Database development**

- Develop better global data, especially in countries outside North America and Europe, on status and trends of environmental contamination, exposure, and health outcomes.
- Improve international coordination for sharing of information on effects caused by endocrine disruption.

This state-of-the-science assessment has revealed that our current understanding of the effects posed by EDCs to wildlife and humans is incomplete. The evidence that high-level exposure may impact both humans and wildlife indicates that this potential mechanism of toxicity warrants our attention. Uncertainty over the possible effects of chronic, low-level exposures to a number of chemicals with endocrine-disrupting potential and the fundamental roles played by the endocrine system in maintaining homeostasis make understanding the potential effects posed by exposure to these chemicals an obvious international priority. There is a need to identify life stages and species that are more vulnerable to the effects of EDCs and to understand how this mechanism of toxicity may affect individual populations and communities.

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Global concerns have been raised in recent years over the potential adverse effects that may result from exposure to chemicals that have the potential to interfere with the endocrine system. Wildlife and human health effects of EDCs were first proclaimed by Rachel Carson in 1962 and based on a growing body of knowledge those concerns have increased. This concern regarding endocrine disrupting chemicals (EDCs) is directed at both humans and wildlife. In response to these concerns, the Second Session (February 1997) of the Intergovernmental Forum on Chemical Safety made a number of recommendations to the Member Organization of the Inter-Organization Programme for the Sound Management of Chemicals (IOMC), notably IPCS and OECD, concerning approaches and means for coordinating and/or supporting efforts to address the issues internationally, including the development of an international inventory of research and coordinated testing and assessment strategies. This endorsed earlier recommendations from an international workshop at the Smithsonian (January 1997) and was followed by the 1997 Declaration of the Environmental Leaders of the Eight on Children's Environmental Health, which specifically addressed the issue of EDCs in their declaration. The environment leaders encouraged continuing efforts to compile an international inventory of research activities, develop an international assessment of the state-of-the-science, identify and prioritize research needs and data gaps, and develop a mechanism for coordinating and cooperating on filling of the research needs. The Fiftieth World Health Assembly adopted resolution WHO 50.13 in 1997, which called upon the Director-General of WHO to "take the necessary steps to reinforce WHO leadership in undertaking risk assessment as a basis for tackling high priority problems as they emerge, and in promoting and coordinating related research, for example, on potential endocrine-related health effects of exposure to chemicals."

In response to these recommendations, the WHO/UNEP/ILO International Programme on Chemical Safety (IPCS) assumed responsibility for developing this global assessment of the current state of scientific knowledge relative to environmental endocrine disruption. Concurrently, IPCS assisted in the development of a Global Endocrine Disruptor Research Inventory, which serves as a tool to foster complementary research efforts and identify strengths and weaknesses of current global research efforts.

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INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among UNEP, ILO, FAO, WHO, UNIDO, UNITAR and OECD