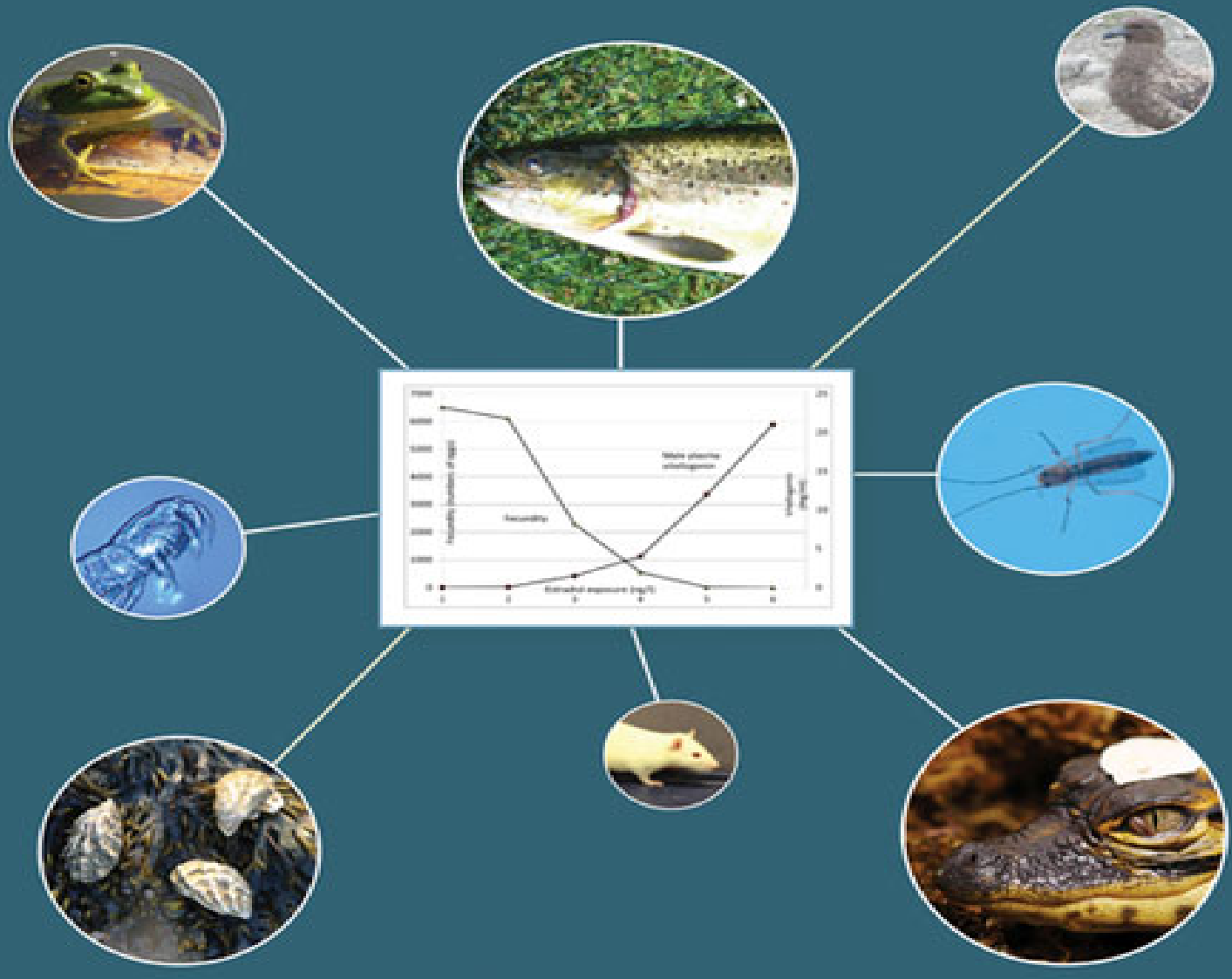
ENDOCRINE DISRUPTERS

Hazard Testing and Assessment Methods



EDITED BY Peter Matthiessen

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HAZARD TESTING AND ASSESSMENT METHODS

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PREFACE

The purpose of this book is to describe the state of the art of *in vivo* ecotoxicological testing methods for endocrine disrupting chemicals. It includes consideration of mammalian methods but addresses *in vitro* techniques in only a few chapters. The book not only covers methods for all the vertebrate groups as well as several invertebrate phyla but also addresses regulatory issues and possible approaches to hazard assessment and prediction. The book is divided into chapters covering each major taxon as well as the regulatory and hazard assessment topics. Every chapter has been written by acknowledged experts in their respective fields. Furthermore, the main chapters have been subjected to peer review and amended in the light of reviewers' comments.

The book does not claim to be exhaustive, although it does provide a helpful route into the subject. This is still a fast-moving field, but the information contained within this volume will be of assistance to scientists involved in testing chemicals for possible endocrine-disrupting properties as well as to regulatory authorities responsible for deciding whether chemicals can be marketed safely. This volume reflects the current (spring 2012) state of knowledge and thus deals largely with those modalities of endocrine disruption that are reasonably well understood and can be broadly categorized as estrogenic, androgenic, thyroidogenic, or steroidogenic (EATS). It must, however, be recognized that environmental chemicals are probably interacting with many other endocrine systems, and as these mechanisms are gradually uncovered, this book will require substantial updating. I nevertheless hope that

readers will find this distillation of current knowledge useful and stimulating.

Peter Matthiessen
March 2012

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CHAPTER 1

Ecotoxicity Test Methods for Endocrine-Disrupting Chemicals

AN INTRODUCTION

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1.1 BACKGROUND

The issue of endocrine disruption has been something of a *cause célèbre* since it was first identified as an issue about 25 years ago. Few scientists had previously suspected that certain synthetic chemicals might be able to interfere with the workings of the endocrine system at low concentrations. However, in the mid-1990s, Theo Colborn and others [1] brought this subject to the attention of a wide audience when it became clear that many different wildlife species were experiencing effects that were attributable to

damaged hormone signaling. Since then, endocrine-disrupting chemicals (EDCs) have come to be treated as a special case rather like carcinogens, so that the mere possession of endocrine-disrupting (ED) properties can be enough to trigger precautionary regulatory action in some jurisdictions, irrespective of the probable environmental risks involved. In other jurisdictions, the risks of EDCs are being evaluated in similar ways to non-EDCs, but these chemicals are the subject of much concern irrespective of the regulatory stance being taken.

It is therefore extremely important that EDCs should be unambiguously identified and their hazards accurately measured. This book represents one of the first attempts to describe and critically evaluate the methods that have been developed for studying the effects of EDCs on mammalian and nonmammalian wildlife in the laboratory.

The chapters in this volume are aimed at scientists and chemical companies that wish to investigate the ecotoxicological properties of EDCs using cutting-edge and (where relevant) internationally agreed techniques and at chemical regulatory authorities that seek to protect the environment from the adverse effects of EDCs through the use of rigorous hazard evaluation programs that employ scientifically sound methods. This is quite an ambitious aim, given that some standardized assays that use wildlife species to assess the toxicity of EDCs are still in development, and there remain whole classes of EDCs for which assays have not yet been standardized or even designed. However, despite these gaps and limitations, it is hoped that the book will provide useful guidance until a more comprehensive array of test methods becomes available.

Chapter 2 by Dick Vethaak and Juliette Legler describes why some EDCs became recognized as an environmental problem in the 1980s, and summarizes the large amount of

research that has since discovered many features of this disparate group of chemicals. The chapter brings us up to date about the chemicals that have been found to have ED properties and surveys effects observed in the field and in the laboratory. This review makes it clear that, until recently, chemical risk assessment schemes had failed to prevent some EDCs from entering the environment and causing sometimes severe damage to certain ecosystems. Thus, the chapter sets the scene for the ones that follow.

1.2 REGULATORY CONCERNS

In Chapter 3, Hans-Christian Stolzenberg and coauthors explain why regulatory authorities have become concerned about EDCs and describe in detail how several authorities (especially Japan, the United States, and the European Union) have responded with new programs and regulations designed to identify these chemicals and assess their hazards and associated environmental risks. It became apparent from the early 1990s that existing internationally standardized ecotoxicity assays were largely insensitive to EDCs. As a result of this understanding, member countries of the Organisation for Economic Cooperation and Development (OECD) initiated a program to develop and validate new testing methods. The chapter indicates how these methods are likely to be used in chemical regulatory activities in several jurisdictions, although full details are still being developed and many other jurisdictions have yet to act.

1.3 INVERTEBRATES

The regulatory background is then followed by three chapters that describe testing methods with certain invertebrate groups (insects, crustaceans and molluscs) and

five chapters covering methods using vertebrates (fish, amphibians, reptiles, birds, and mammals). At present, regulatory requirements for the testing of suspected EDCs are restricted to vertebrates alone, but this is due mainly to the fact that invertebrate endocrine systems are relatively poorly understood, not because endocrine disruption is not an issue in these phyla.

Chapter 4 by Lennart Weltje concerns testing methods in insects. The endocrine systems of insects, of all the invertebrates, are the best understood due to their overwhelming importance as pests, a fact that has led to the development of pesticides specifically intended to cause endocrine disruption in this group. The chapter not only describes *in vivo* testing methods covering key endocrine-mediated processes such as growth and reproduction but also a range of *in silico* and *in vitro* mechanistic techniques that show promise for the understanding of certain modes of action. This aspect is important given that generally agreed definitions of EDCs require that an apical effect *in vivo* needs to be plausibly linked to an ED mechanism.

Crustacean test methods are covered by Magnus Breitholtz in Chapter 5. This invertebrate group belonging to the arthropods shares many endocrine similarities with insects and is also economically important, but in this case as a food source. The chapter goes into the endocrinology of various crustacean taxa in considerable detail, and it is clear that a range of mechanistic assays will be developed in the near future. At present, however, available methods include several with *in vivo* apical endpoints (especially reproductive success) which do not in themselves reveal modes of action. The chapter also includes consideration of some newer techniques including toxicogenomic methods which show promise for the future.

In Chapter 6, Patricia D. McClellan-Green addresses possible endocrine testing methods involving molluscs. In

comparison with the invertebrate groups discussed in Chapters 4 and 5, less is known about the endocrine systems in this phylum, although there is good evidence that endocrine disruption can be caused by a variety of substances, some of which (e.g., organotins) are much more potent than in other phyla. For this reason, *in vitro* techniques are still in their infancy, and we are not yet in a position to standardize mechanistic *in vivo* molluscan screens, although some biomarkers (e.g., vitellogenin and imposex induction) show promise. Perhaps surprisingly, no mollusc-based toxicity tests of any kind have yet been internationally standardized, but an OECD project led by the United Kingdom, Germany, France, and Denmark is now developing partial and full life cycle apical tests with gastropods that will be useful for the assessment of both EDCs and non-EDCs.

1.4 VERTEBRATES

Throughout the evolution of the vertebrates, there has been a high degree of conservation of their endocrine systems, with many hormones and receptors being identical or very similar across the vertebrate groups. However, despite these similarities, tests with sensitivity to EDCs are needed for most of the major vertebrate groups because of differences in exposure, metabolic competence, and downstream hormonal interactions. Chapters 7 to 11 address methods involving all vertebrate groups from fish to mammals.

Peter Matthiessen discusses toxicity tests for EDCs using fish in Chapter 7. Some of the earliest widespread effects of EDCs observed in the field involved this group of vertebrates (feminization of male fish exposed to estrogens), and considerable progress has been made in developing and standardizing fish-based test methods with

sensitivity not only to (anti)estrogens but also to (anti)androgens and steroidogenesis disrupters. Three different fish-based screening assays have now been published by OECD that are able to provide mechanistic information about the potential of a chemical to interfere with different aspects of the steroid hormone system *in vivo*, and one of these also provides some apical information about possible effects on reproductive success. A partial life cycle test (the Fish Sexual Development Test) has also been published, providing mechanistic and apical information concerning possible impacts on phenotypic sex ratio. Another partial life cycle test covering the reproductive phase of the life cycle is in development, as are full and multiple life cycle tests. When these are complete, a comprehensive suite of tests for EDCs using fish will be in place.

Chapter 8 by Daniel B. Pickford covers testing of EDCs using amphibians. This group is particularly sensitive to thyroid system disrupters, and the chapter goes into detail about the development and standardization of larval-based screens that are responsive to these chemicals. An amphibian *in vivo* mechanistic screen (the Amphibian Metamorphosis Assay) has now been published by OECD and shows sensitivity to several different types of thyroid interference. The chapter then goes on to describe possible partial and full life cycle testing in this group, although standardization of such higher-tier tests is still ongoing. Research has already shown that the sexual development of some amphibians can be disrupted by exposure to several different types of EDC including estrogens. However, at present there are no plans to standardize full life cycle tests with amphibians due to the difficulty and expense of culturing the currently used species in the laboratory.

Chapter 9 by Satomi Kohno and Louis J. Guillette Jr. discusses reptiles, for which no internationally standardized

tests for EDCs are currently being considered. Reptiles have not traditionally been used in ecotoxicity tests, but several members of this group possess an interesting physiological trait that can be exploited to study endocrine disrupters. In brief, the sex of many young reptiles (e.g., turtles and alligators) is determined by the temperature at which the eggs are incubated, and this process can be subverted by certain EDCs. For example, in crocodilians, lower temperatures produce females alone, intermediate temperatures produce both sexes, and higher temperatures produce males alone; administration of low estrogen doses at male-producing temperatures leads to the induction of females. The chapter describes both the use of estrogen receptor transactivation assays that employ receptors derived from reptiles to measure estrogenic activity *in vitro* and *in vivo* assays that exploit interference with sex determination in species such as the American alligator. The drawback of the *in vivo* methods is that reptile eggs are generally produced only seasonally and are available commercially in relatively small numbers, which may explain why there has been no attempt at standardization to date.

Testing for EDCs using birds is considered in Chapter 10 by Paul D. Jones, Markus Hecker, Steve Wiseman, and John P. Giesy. Life cycle characteristics such as egg laying may make birds particularly sensitive to some EDCs, although the avian endocrine system has many similarities with those of other higher vertebrates. However, although the mechanism of sex determination is not fully understood, it is known that estradiol is the sex-differentiating hormone in birds (testosterone plays this role in mammals), so administration of estrogens to birds during development may cause more profound changes than in mammals. This chapter covers *in vitro* techniques with avian cell lines and *in vivo* methods using both embryos and adult birds. Dosing

methods comprise egg injection and feeding, and studies can include both partial and full life cycles. An avian partial life cycle reproduction test was published by OECD many years ago, but an avian two-generation test is currently being validated by that organization, and aspects of the test are considered in this chapter.

Chapter 11 by M. Sue Marty covers methods for studying endocrine disruption in mammals. Due to the importance of mammalian tests for predicting chemical effects in humans, they have been more extensively developed than those with lower vertebrates, although some with particular sensitivity to EDCs were standardized and published only recently. This chapter describes an array of five standardized mammalian tests with rodents that can be used to identify ED activity *in vivo* and indicates how they can be integrated into a screening program for estrogens, androgens, and thyroid-acting compounds. Consideration is then given to more extended rodent-based assays (the two-generation and extended one-generation tests). which could be used at a higher level of testing in order to reveal a fuller range of possible apical effects. The chapter concludes with a discussion of the relevance of these tests for predicting the effects of EDCs in humans and mammalian wildlife.

1.5 TESTING SCHEMES FOR EDCS

Chapter 12 by Thomas H. Hutchinson, Jenny Odum, and Anne Gourmelon describes the five-level OECD Conceptual Framework (CF) that was developed to guide the standardization of screens and tests for EDCs. The CF levels move from Level 1 (data gathering), through Level 2 (*in vitro* assays), to Levels 3 to 5 covering *in vivo* assays of increasing complexity. Standardized *in vivo* assays for both mammals and nonmammals are now available at each of Levels 3 to 5, but it is clear that these levels are not

necessarily to be followed in a linear testing scheme. The authors explain why a weight-of-evidence approach is required to assess whether substances have ED properties, and if so, whether those properties are able to cause adverse apical effects. They use two case studies to illustrate how weight-of-evidence assessments might work using the assays in the CF and conclude that the framework provides a logical process for critically evaluating studies that show either positive or negative results. Overall, the assays in the CF are found to provide useful data for identifying EDCs and measuring the type and magnitude of their effects in mammals and other wildlife.

In the final chapter (Chapter 13), Peter Matthiessen continues the discussion of possible testing approaches for EDCs and reiterates the need for weight-of-evidence assessments. Remaining gaps in the testing tool box are identified, but it is clear that a reasonably complete set of assays for so-called EATS modalities (i.e., EDCs with estrogen/androgen/thyroid/steroidogenic action) will be available within the next few years. However, current standardized testing procedures do not account for several new types of endocrine disruption that will need consideration in due course. The chapter then discusses possible integrated procedures for testing EDCs and presents a draft scheme for assessing the hazards posed by chemicals to fish recently discussed at an OECD workshop. This scheme covers all chemicals, not just EDCs, and attempts to integrate the new fish-based tests for EDCs into a wider framework.

To summarize this book, it is clear that the development and standardization of ecotoxicity tests for EDCs remains a work in progress, but great strides have been made during the first decade of the twenty-first century. Enough validated assays are now in place (or will shortly be agreed) to permit the routine operation of hazard assessment

schemes for suspected EDCs, providing that the limitations of these assays are borne in mind.

REFERENCE

1. Colborn, T., Dumanoski, D., Myers, J. P. (1996). Our Stolen Future. Penguin Books, New York. 306 pp.

CHAPTER 2

Endocrine Disruption in Wildlife

BACKGROUND, EFFECTS, AND IMPLICATIONS

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2.1 BACKGROUND TO ENDOCRINE DISRUPTION

2.1.1 Introduction

It is now well recognized that endocrine-disrupting chemicals (EDCs) pose a potential risk affecting both wildlife and human health on a global scale [1,2]. EDCs are generally defined as substances in our environment, food, and consumer products that can disrupt hormonal balance and result in adverse health effects. An EDC elicits adverse health effects primarily by its interaction with an endocrine mechanism (endocrine disruption), given the right dose and timing of exposure. In the last decades, field and laboratory studies have shown that some EDCs, including natural hormones, pharmaceuticals, some pesticides, and industrial contaminants, can cause developmental, reproductive, neural, immune, and other problems in a range of wildlife taxa [1,3–11]. Nowadays there is clear evidence that a growing number of environmental chemicals may possess endocrine-disrupting (ED) activity and that ED effects can occur at very low concentrations, concentrations that are

similar to current exposure levels. ED effects may occur at much lower doses than other types of toxicants that act through different mechanisms. In addition to having adverse effects on wildlife, there are growing indications for associations of certain persistent organic pollutants (POPs) with ED effects in humans at the relatively low doses typically found in the environment—for example, male testicular dysgenesis syndrome (lower sperm count; increases in hypospadias [urogenital abnormalites in male babies], and cryptorchidism [the absence of one or both testes from the scrotum]) and certain cancers [12–14]. Recent research also links human EDC exposure with obesity, metabolic syndrome, and type 2 diabetes (reviewed by [15–17]). The scope and magnitude of EDC harm to wildlife populations and possibly to humans are becoming increasingly apparent, as our knowledge and understanding increase, and demonstrate the need for prompt policy actions and the need for regulation and testing of EDCs.

In this introduction and background chapter, we provide—at the risk of repeating earlier publications—a general description of the issue of endocrine disruption in the environment with particular reference to wildlife. We do not intend to be complete (which is almost impossible given the rapid development in this field), but instead we focus on new developments, the wildlife–human connection, and implications for field monitoring and effect assessment and toxicity testing.

2.1.2 Brief Account of the History of Endocrine Disruption

The effects of EDCs have been evident since the 1940s [18]. Originally reported in peregrines (*Falco peregrinus*), around 1946, DDE-induced reproduction failure due to eggshell thinning and broken eggs has been described in a large

number of raptor species [19]. This is probably also the first example of endocrine disruption in wild populations, even though a plausible mechanism of action has not been elucidated until recently (reviewed by [20]). Since the appearance of Rachel Carson's *Silent Spring* in 1962 [21] and the diethylstilbestrol (DES) story [22], the public has been increasingly concerned about those chemicals that could have harmful effects on wildlife and human health. The potential environmental impacts of synthetic hormones, industrial by-products, pesticides, and other chemicals introduced to the environment led in 1979 to the start of a successful and still-continuing series of conferences on estrogens in the environment organized by the U.S. National Institute of Environmental Health Sciences (NIEHS) [23–25]. In 1991, a group of expert scientists at the Wingspread Conference titled "Chemically Induced Alterations in Sexual Development: The Wildlife/Human Connection" presented a review of the scientific literature from the 1950s through the 1980s, where they found a wide number of pathologies and other abnormalities in wildlife and humans that seemed to be connected to endocrine system dysfunction. A consensus statement released from that group stated:

> Many compounds introduced into the environment by human activity are capable of disrupting the endocrine system of animals, including fish, wildlife, and humans. The consequences of such disruption can be profound because of the crucial role hormones play in controlling development. [26, page 1].

This alarming statement of the potential dangers posed by EDCs provided a major impetus for further studies and the discovery of numerous new cases of adverse effects of EDCs in wildlife and humans worldwide. In 1996, Theo Colborn and coworkers Dianne Dumanoski and John Peterson Myers published *Our Stolen Future* [27] and introduced the endocrine disruptor hypothesis, in which they pointed out