



# Advancements in Endocrine Disruptors Research

SUMMARY OF U.S. EPA  
ACCOMPLISHMENTS 1996-2007

**Draft**



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The information presented in this accomplishments report is intended to provide the reader with insights about the progress and scientific achievements of the Endocrine Disruptors Research Program. This report is not sufficiently detailed nor is it intended to be used directly for health or environmental assessments. Readers with these interests should instead consult the peer reviewed publications and conduct necessary data quality evaluations as required for their assessments.

Throughout the report ORD describes research that it has conducted either on endocrine disrupting chemicals, in general, or on specific chemicals or classes of chemicals. It should not be construed that just because ORD is studying specific chemicals or classes of chemicals that this means that EPA has determined that these chemicals or classes are officially designated as “endocrine disruptors.” Those determinations will be made by EPA through the implementation of the Endocrine Disruptor Screening Program.

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## EXECUTIVE SUMMARY

In 1996, the U.S. Environmental Protection Agency (EPA) Office of Research and Development (ORD) initiated an Endocrine Disruptors Research Program to provide the scientific information needed to reduce or prevent unreasonable risks to humans and wildlife from exposures to individual pesticides and toxic chemicals and environmental mixtures of chemicals that interfere with the function of the endocrine system. It has been suggested that humans and domestic and wildlife species have suffered adverse health consequences resulting from exposure to chemicals in the environment that interact with the endocrine system. However, considerable uncertainty exists regarding the relationship(s) between adverse health outcomes and exposure to environmental contaminants. Collectively, chemicals with the potential to interfere with the function of endocrine systems are called endocrine disruptors or endocrine disrupting chemicals (EDCs). EDCs have been defined as exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of the natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes

Despite a range of effects observed primarily in wildlife species with relatively high exposures to specific compounds, we know little about their causes and the concentrations of EDCs that would induce effects at the population level. Nevertheless, it is known that the normal functions of all organ systems are regulated by endocrine factors. Small disturbances in endocrine function, especially during certain stages of the life cycle such as development, pregnancy, and lactation, can lead to profound and lasting effects. Research on endocrine disruptors was identified as one of the six high-priority topics in the ORD Strategic Plan. This was based upon recognition of: (1) the potential scope of the problem, (2) the possibility of serious effects on the health of populations, (3) the persistence of some endocrine-disrupting agents in the environment, and (4) the widespread global concern about the fate and transport over national borders.

ORD's Endocrine Disruptors Research Program is designed around the risk assessment/risk management paradigm and strikes a balance between "problem-driven" (understanding and solving particular, identified environmental problems) and "core" (improving the basic underlying science) research. The objectives of the EDCs research program are to improve our knowledge and understanding of endocrine disruptors in the environment so that we can improve our methods of assessment and risk management. This, in turn, will assist EPA in identifying the chemicals that pose an unreasonable risk, developing ways to prevent or reduce their release into the environment, and developing means to remediate in-place EDCs that pose an unreasonable risk. Results from the research program are used by EPA's Office of Prevention, Pesticides, and Toxic Substances, Office of Air, and Office of Water in developing and implementing regulations and providing improved risk assessment and management of EDCs.

The EDRP addresses three overarching science needs that are established as the long-term goals for the research program in the Research Plan and Multi-Year Plan. These long-term goals are:

- Reduction in uncertainty regarding the effects, exposure, assessment, and management of endocrine disruptors so that EPA has a sound scientific foundation for environmental decision-making.
- Determination of the extent of the impact of endocrine disruptors on humans, wildlife, and the environment to better inform the federal and scientific communities.

- EPA's Office of Prevention, Pesticides, and Toxic Substances using endocrine disruptors screening and testing assays developed by ORD to create validated methods that evaluate the potential for chemicals to cause endocrine-mediated effects in order to reduce or prevent risks to humans and wildlife from exposure to endocrine disruptors.

EPA is a leader in endocrine disruptor research. No other programs have similar goals, in terms of scope and mission, as the EDRP, and no other single organization has such an extensive portfolio of research regarding EDCs. Research conducted through the EDRP has been instrumental in developing test methods, understanding the potential effects of EDCs on early development and childhood, and characterizing the exposure and effects of EDCs in wildlife. As a result, uncertainties have been reduced and EPA now has the ability to understand and effectively assess the relationship(s) between exposure to environmental contaminants and adverse health outcomes. The methods, models, and data developed through the EDRP are externally peer-reviewed and widely disseminated.

The products of this research provide a scientific foundation for standardized and validated screening and testing protocols; interpreting toxicity and exposure data on EDCs; identifying risk management approaches for EDCs, and general decision-making related to EDCs. Progress is measured by the extent to which methods, models, and/or data from the EDRP are actually used in risk assessments and other analyses supporting decisions. The use of EDCs research products by EPA and others will improve the scientific basis of actions related to reduction or prevention of exposures or releases of potentially harmful EDCs into the environment.

The Endocrine Disruptors Research Program includes intramural research conducted at EPA's national laboratories and extramural research supported through the Science to Achieve Results (STAR) competitive, peer-reviewed grants program. Since 1998, the total of funds spent on EDC research is \$117.5 million; of that total \$90 million has supported intramural research and \$27.5 has gone to extramural research. Those expenditures have funded 51 grants funded at 36 institutions in 25 states. Within EPA, coordination of intramural and extramural programs has ensured efficient use of available funds and development of and access to top-quality scientists necessary for addressing the program's challenging research questions. External to EPA, there is close cooperation and collaboration on endocrine disruptors research across the federal government and internationally because the key uncertainties regarding endocrine disruptors are so complex, interests across agencies and organizations overlap, and resources are limited.

The program makes best use of resources and encourages cooperation with other organizations with similar interest in EDCs and public health. The research has resulted in (1) the completion of standardization and prevalidation studies for screening and testing assays, (2) internationally expressed interest in the use of the protocols, and (3) invitations to ORD and STAR grant investigators to serve on national and international workgroups and committees to develop harmonized guidelines and methods for detecting EDCs. Through its focused federal funding and contributions from program partners, plus the drawing together of researchers from prestigious institutions, the program has multiplied many times over the body of knowledge, screening and testing techniques, and depth of awareness of multigenerational hazards chemicals can have on humans, domestic animals, and wildlife.

The Endocrine Disruptors Research Program has led to significant progress in identifying sources of EDCs, characterizing their environmental concentrations, and assessing their potential to cause adverse effects. Research has identified and begun characterizing sources of EDCs

including surface water discharges from wastewater treatment plants, concentrated animal feeding operations, and pulp and paper mills. The relationships between pollutant exposures during critical developmental stages and adverse outcomes later in life including influence on reproductive health (e.g., reproductive tract development, sexual maturation, and fertility) and other systemic effects such as on thyroid, nervous, and immune dysfunction have been investigated. Investigators are developing new laboratory models for studying how endocrine disruption might occur during early development.

The extensive research has included wildlife species that may be especially vulnerable to the effects of endocrine disruptors for several reasons: (1) They have potential for exposure to high levels of endocrine disruptors in soils, sediments, surface waters, and through their diets; (2) They have particular species-specific biological sensitivities to EDCs; (3) The differences in their endocrine systems may cause effects that differ from the systems of laboratory animals, therefore complicating extrapolation from laboratory to field; and (4) Their exposure to multiple EDCs makes it especially difficult to characterize the toxic effects from mixtures of EDCs.

This report summarizes scientific accomplishments achieved by the program since its inception roughly 10 years ago. Chapter 1 gives an overview of the program including its goals, partnerships, history, indicators of its performance, and impacts of the research. The remaining chapters summarize accomplishments in three main topic areas: screening and testing (chapter 2), early developmental exposure and consequences (chapter 3), and wildlife exposure and effects (chapter 4).

It should be noted that throughout the report, ORD describes research that it has conducted either on EDCs, in general, or on specific chemicals or classes of chemicals. It should not be construed that just because ORD is studying specific chemicals or classes of chemicals that this means that EPA has determined that these chemicals or classes are officially designated as “endocrine disruptors.” Those determinations will be made by EPA through the implementation of the Endocrine Disruptor Screening Program.



# CHAPTER 1. RESEARCH PROGRAM OVERVIEW

Endocrine disruptors or endocrine disrupting chemicals (EDCs) are exogenous substances that interfere with the endocrine system and can disturb the physiologic functions of natural hormones in the body. In the last 2 decades, scientific studies have established that exposure to EDCs has the potential to cause adverse health effects by altering the function of the endocrine system. Potential effects can include developmental malformations, interference with reproduction, increased cancer risk, and disturbances in immune and nervous system function.

EPA is a leader in endocrine disruptor research. Research conducted or supported by EPA's Office of Research and Development (ORD) has been instrumental in developing test methods, understanding the potential effects of EDCs on early development and childhood, and characterizing the exposure and effects of EDCs in wildlife. As a result, uncertainties have been reduced, and EPA has an improved ability to understand and effectively assess the relationship(s) between exposure to environmental EDCs and adverse health outcomes.

## Why does EPA have an endocrine disruptors research program?

Growing concern for EDCs in the environment in the early 1990s led the Federal government to take legislative action. In 1996, Congress enacted the Food Quality Protection Act (FQPA), and directed EPA to screen pesticides for estrogenic activity in humans using validated studies or other scientifically relevant information with discretionary authority to screen for other endocrine effects. The Safe Drinking Water Act (SDWA) Amendments of 1996 also authorized EPA to screen drinking water contaminants for endocrine activity. The EPA's Office of Research and Development (ORD), in order to address existing research gaps, integrated and expanded its EDC research efforts into a consolidated Endocrine Disruptors Research Program.

The program increases knowledge and understanding of endocrine disruptors in order to improve risk assessment and management. ORD's Endocrine Disruptor Research Program conducts both basic and applied research to develop the fundamental scientific principles used by the EPA program and regional offices in making risk assessment decisions.

### What are Endocrine Disruptors?

Kavlock et al. published the EPA definition of an endocrine disruptor as "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body which are responsible for the maintenance or homeostasis, reproduction, development and or behavior."<sup>a</sup>

<sup>a</sup>Kavlock RJ, Daston GP, DeRosa C, Fenner-Crisp P, Gray LE, et al. 1996. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored workshop. Environmental Health Perspectives 104(S-4):1-26.

"The objectives of the Endocrine Disruptors Research Program are to improve our knowledge and understanding of endocrine disruptors in the environment so that we can improve our methods of assessment and risk management.

This, in turn, will assist the Agency in identifying the chemicals that pose an unreasonable risk, developing ways to prevent or reduce their release into the environment, and developing means to remediate in-place EDCs that pose an unreasonable risk."

— EPA ORD's Multi-Year Plan (FY2000-2012) for Endocrine Disruptors, December 2003

## Disruption of the Endocrine System

Potential human health effects that could result from the disruption of the endocrine system include breast cancer and endometriosis in women, testicular and prostate cancers in men, abnormal sexual development, reduced male fertility, alteration in pituitary and thyroid gland functions, immune suppression, and neurobehavioral effects.<sup>a</sup>

Effects in wildlife and aquatic organisms proposed to be associated with endocrine disruption mechanisms include abnormal thyroid function and development, decreased fertility, decreased hatching success, decreased survival of offspring, feminization, masculinization, and alteration of immune and behavioral function.<sup>a</sup>

Chemicals can disrupt estrogen, testosterone, and thyroid hormone systems. Examples include the following:

- Estrogen mimic: **ethinyl estradiol**, an active ingredient found in oral contraceptives, binds with and activates the estrogen receptor.
- Androgen mimic: **flutamide**, a drug used to treat prostate cancer, is metabolized by the body to an active compound that binds with and stimulates androgen receptors.
- Thyroid hormone disruptor: **perchlorate**, a chemical used in solid rocket fuels and found in environment as a drinking water contaminant binds with and blocks the action of a protein that transports iodide into the thyroid, thereby decreasing synthesis of the thyroid hormones T<sub>4</sub> and T<sub>3</sub>.

<sup>a</sup> U.S. EPA. 1997. Special Report on Endocrine Disruptors: An Effects Assessment and Analysis.

Endocrine disruptors are a research priority for EPA because of (1) the potential scope of the problem; (2) the possibility of serious health effects on populations; (3) the persistence of some EDCs in the environment; and (4) the widespread global concern about the fate and transport of EDCs across national borders.<sup>1</sup>

In 1996, EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to advise the Agency on implementing the provisions of the amendments to the Safe Drinking Water Act and the Food Quality Protection Act. This activity is being carried out by the EPA's Endocrine Disruptor Screening Program, which is managed by the Office of Prevention, Pesticides, and Toxic Substances (OPPTS). The efforts are supported by research conducted in the ORD's Endocrine Disruptor Research Program. Additionally, in response to the 1996 Congressional mandate that EPA develop a screening and testing program for water and food contaminants, EPA needed to develop and validate new methods for assessing chemicals for "estrogenic or other endocrine effects in humans." While some potentially suitable assays existed, extensive research has been conducted to validate and standardize methods.

### What are the goals of the research program?

ORD's 1998 Strategic Research Plan for Endocrine Disruptors identified key areas of uncertainty and provided an overarching road map of prioritized research needs to improve EPA's understanding of endocrine disruptors.<sup>2</sup> The 2003 Multi-Year Plan (FY2000–2012) for Endocrine Disruptors set three long-term program goals, and set annual performance goals and measures for the program.<sup>3</sup>

## ORD Long-term Goals for the Endocrine Disruptors Research Program

Long-Term Goal	Key Areas of Uncertainty Addressed by Goal
1. Reduction in uncertainty regarding the effects, exposure, assessment, and management of endocrine disruptors so that EPA has a sound scientific foundation for environmental decision-making	<ul style="list-style-type: none"> <li>– What are dose-response characteristics in the low-dose region?</li> <li>– What extrapolation tools are needed?</li> <li>– What are effects of exposure to multiple endocrine disruptors, and will a toxic equivalency factor (TEF) approach be applicable?</li> <li>– How can unreasonable risks be managed?</li> <li>– What approaches are needed to assess risks to humans and wildlife?</li> </ul>
2. Determination of the extent of the impact of endocrine disruptors on humans, wildlife, and the environment to better inform the federal and scientific communities	<ul style="list-style-type: none"> <li>– How and to what degree are human and wildlife populations exposed to EDCs?</li> <li>– What effects are occurring in exposed human and wildlife populations?</li> <li>– What are the chemical classes of interest and their potencies?</li> <li>– What are the major sources and environmental fates of EDCs?</li> </ul>
3. OPPTS using endocrine disruptors screening and testing assays developed by ORD to create validated methods that evaluate the potential for chemicals to cause endocrine-mediated effects in order to reduce or prevent risks to humans and wildlife from exposure to endocrine disrupting chemicals	<ul style="list-style-type: none"> <li>– Do our testing guidelines and methods adequately evaluate potential endocrine-mediated effects?</li> </ul>

EPA's Endocrine Disruptors Research Program is one of only a few ORD programs that focus on multidisciplinary research for *both* human health and wildlife. The research has effectively addressed every aspect of the risk assessment and risk management paradigm:

- Determined *sources* of EDCs and *exposures* to humans and ecosystems.
- Developed *integrated risk assessment approaches* for human and wildlife populations.
- Developed *risk management approaches* to mitigate exposures in human and wildlife populations.

Program research has assessed effects and exposures of endocrine disruptors for a diverse group of organisms including invertebrates, fish, frogs, rodent laboratory models, and humans. The program has examined effects at the molecular, cellular, organ, organ system, whole organism, and population levels.

The program strengths are the integration of intramural EPA scientists with extramural research communities. Intramural research is conducted by EPA staff and contractors at three laboratories and one center:

- National Health and Environmental Effects Research Laboratory (NHEERL)

- National Exposure Research Laboratory (NERL)
- National Risk Management Research Laboratory (NRML)
- National Center for Environmental Assessment (NCEA)

The most highly-qualified scientists and engineers in academia and other nonprofit research institutions conduct extramural EDC research through the competitive, peer-reviewed Science to Achieve Results (STAR) program managed by the National Center for Environmental Research (NCER).

## **How has the Endocrine Disruptor Research Program evolved over the past 10 years?**

In its 10 years as a distinct program, the Endocrine Disruptor Research Program has established EPA as a leader in endocrine disruptor research worldwide. EPA has achieved this distinction through consistent strength in leadership and ground-breaking research.

**Consolidated Research.** In 1995, EPA integrated individual ORD scientists conducting endocrine-related research throughout EPA and expanded its research efforts into a consolidated program. At its inception, EPA held two international workshops to develop a national research plan for EDCs and published the proceedings.<sup>4,5</sup>

**Strategic Plan.** ORD developed a strategic Research Plan for Endocrine Disruptors starting in 1995 and published it in 1998. The plan incorporated expert recommendations from the workshops, internal and external peer review comments, and input from the ORD Science Council and ORD Research Planning Committee.<sup>6</sup> The document has served as a roadmap and benchmark for the Agency's EDC research and a guide for international coordination of EDC research.

**Interim Assessment.** In February 1997, ORD teamed with program offices in EPA's Risk Assessment Forum to develop an interim assessment and guidance on EDCs until the National Academy of Sciences (NAS) could complete a more extensive analysis. ORD's Interim Assessment provided a critical overview of the state of the science for environmental endocrine disruption in humans, laboratory testing, and wildlife species.<sup>7</sup>

**Appointment of National Program Director.** In 1999, ORD appointed a National Program Manager to manage the Endocrine Disruptors Research Program. Later that year, ORD elevated the position to National Program Director to emphasize coordination of national and international EDC research efforts.

**Multi-Year Plan.** In 2003, ORD published the Multi-Year Plan (FY2000–2012) for Endocrine Disruptors that identified critical research needs to support the Agency in carrying out its legislative EDCs mandates.<sup>8</sup> The Plan was written with input from EPA program and regional offices and outlined specific research goals for ORD's laboratories and centers based on

## Milestones in the History of EPA's Endocrine Disruptor Research Program



uncertainties identified in the 1998 Research Plan for Endocrine Disruptors. The plan will be updated in 2007.

**Extramural Research.** Since 1995, EPA's competitive, extramural STAR program has issued seven requests for applications (RFAs) for EDC research. The first two RFAs in 1995 and 1996 funded general research on EDCs. Subsequent RFAs have focused on specific topics such as effects on wildlife and human populations, epidemiologic approaches, low-dose effects and modeling, methods to estimate environmental exposure and occurrence, and fate and effects of hormones in animal waste. Two additional RFAs in 2002 and 2003 were developed in cooperation with the EPA Computational Toxicology Program and investigated high-throughput screening and systems biology approaches to develop predictive models for EDCs. Annual progress review workshops have been held by ORD to review progress of individual research projects, and the workshop proceedings are published on EPA's website.<sup>9,10</sup>

**Independent Peer Reviews.** In 2004, the Endocrine Disruptors Research Program underwent the Office of Management and Budget's (OMB) Performance Assessment Rating Tool (PART) review. An independent expert review by EPA's Board of Scientific Counselors (BOSC) was conducted in December 2004. Results of these reviews are described on page 8.

## What partnerships contribute to the success of the EDC research program?

The breadth, complexity, and global importance of endocrine disruptor issues necessitate coordinated and cooperative research efforts within EPA and among U.S. agencies and international organizations. EPA has taken the lead in coordinating endocrine disruptors research and pursuing collaborative research efforts within its own laboratories and centers and with other federal agencies to more quickly and efficiently advance scientific understanding. .

**Cooperation and Collaboration within EPA.** EPA's program and regional offices participate in an Endocrine Disruptor Research Coordination Team and are committed to achieving the research program's annual and long-term goals by working through ORD's Research Planning Process. EPA's Office of Pollution Prevention and Toxics, Office of Water, and regions participate in ORD solicitation planning and review. In the award process ORD, program, and regional offices participate in the review of STAR applications to ensure funding of the highest priority EDC research projects and integration. Potential collaborative projects are identified.

**Federal government agency partnerships.** As part of the President's National Science and Technology Council, Committee on Environment and Natural Resources (CENR), EPA chairs the interagency Endocrine Disruptors Working Group. This working group created a framework for Federal research on EDCs, inventoried Federal research on EDCs, and identified high priority information needs and opportunities for collaboration. CENR is currently updating the EDC research inventory. EPA recently partnered with the U.S. Geological Survey (USGS) for the CENR Federal Interagency Workshop on Endocrine Disruption in the Environment in February 2007. EPA has partnered with other Federal agencies to fund EDC research. For example, in 2000, NIOSH, EPA, NIEHS, and NCI announced a joint research program and awarded 12 grants totaling almost \$19 million for epidemiologic studies on the effects of exposure to endocrine disruptors. This interagency effort represents the largest portfolio of coordinated federally funded research on the impacts of endocrine disruptors on humans.

**Federal Interagency Workshops.** In 2007, EPA and USGS cosponsored a Federal Interagency Workshop on Endocrine Disruption in the Environment (1) to determine the progress in addressing research needs identified in 1996 CENR document on *The Health and Ecological Effects of Endocrine Disrupting Chemicals: A Framework for Planning*; (2) to provide an overview of current federal activities on EDC

### CENR Endocrine Disruptors Working Group Members

Agency for Toxic Substances and Disease Registry (ATSDR)  
Centers for Disease Control and Prevention (CDC)\*  
Department of Defense (DoD)  
Department of Energy (DOE)  
U.S. Geological Survey (USGS)\*  
EPA  
Food and Drug Administration (FDA)  
National Institute of Environmental Health Sciences (NIEHS)\*  
National Institute for Occupational Safety and Health (NIOSH), part of the Centers for Disease Control and Prevention\*  
National Science Foundation (NSF)  
U.S. Department of Agriculture (USDA)  
National Cancer Institute (NCI)\*  
National Oceanic and Atmospheric Administration (NOAA)\*  
Office of Science and Technology Policy  
Smithsonian Institution

\* Jointly funded EDC research grants in collaboration with ORD.

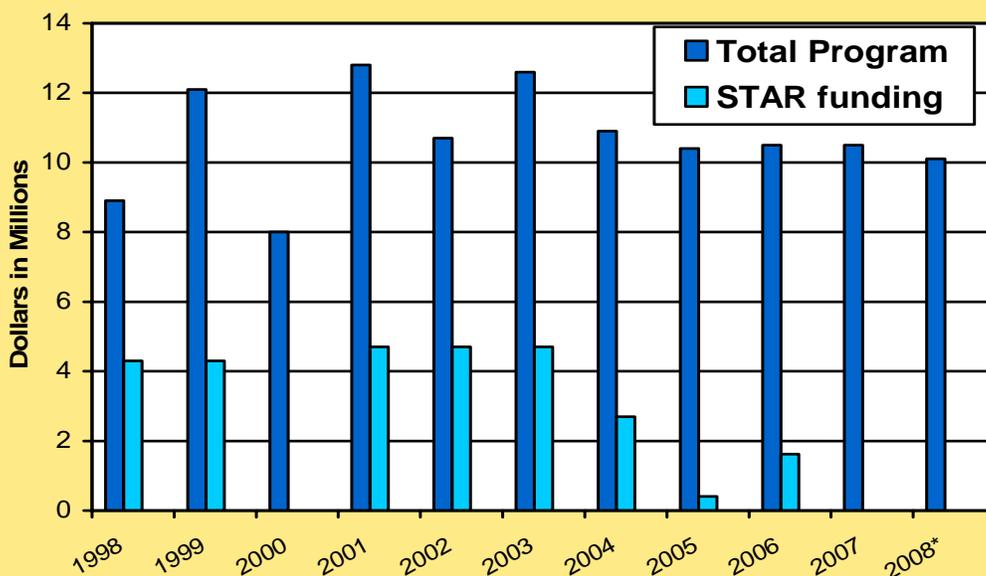
research; and (3) to identify specific areas of collaboration in EDC research and monitoring. Outcomes of the workshop include communication, identification of potential areas of collaboration, an updated inventory of federal EDC research, a summary report, and bibliography.

**International collaboration.** EPA partners with the World Health Organization (WHO) International Programme on Chemical Safety (IPCS), the European Union (EU), the Organization for Economic Cooperation and Development (OECD), and Japan. ORD chaired a steering committee for EDCs comprising WHO, IPCS, and OECD and provided funding and expert support to develop a WHO international assessment of the state of the science of endocrine disruptors in 2002. ORD developed the Global Endocrine meetings, and ORD staff chaired an international workshop, “Endocrine Disruptors: Research Needs and Future Directions,” hosted by Japan’s Ministry of Environment and WHO in December 2003.<sup>11</sup> ORD participates in OECD work groups to develop validated assays, particularly for ecotoxicity tests for a research program that supports U.S. researchers studying wildlife populations in other countries. The OECD work groups continue to collaborate with the Global Water Research Coalition.

### Program Funding

The Endocrine Disruptors Research Program funding averaged \$11 million a year and ranged from \$8 to \$12.8 million a year from 1998 to 2007.

EPA has invested significant funds in the research since 1998: \$107.4 million (\$80 million on intramural research and \$27.4 million on extramural research) for 52 grants at 36 institutions in 25 states. Funding is augmented with additional funds from partners and maximized through the integration of intramural and extramural programs.



Note: Annual funding levels reflect total enacted budget including payroll, travel, and operating expenses.

## Is the Endocrine Disruptor Research Program successful in meeting its goals?

EPA's Endocrine Disruptor Research Program performs highest quality research. Measures of the program's performance include peer review by independent expert panels, the number of peer-reviewed publications generated by the research program, the impact of these publications as indicated by a bibliometric analysis, and the use of research results in risk assessment documents or regulatory decisions.

In 2004, OMB evaluated EPA's Endocrine Disruptor Research Program and regulatory program with the PART review process.<sup>12</sup> The PART review is designed to evaluate Federal programs to ensure accountability, identify strengths and weakness, and suggest improvements. The PART assessment rated EPA's Endocrine Disruptors Research Program as performing *adequate* overall. It was judged to be strong in effective collaboration and coordination with other related programs, budget prioritization, mission clarity, and timeliness of subject matter.

**Results of Independent Peer Review.** The Endocrine Disruptor Research Program has been reviewed by separate panels of expert scientists including the EPA Board of Scientific Councilors (BOSC) and the National Academy of Sciences (NAS).

The EPA BOSC is a public advisory committee chartered under the Federal Advisory Committee Act to provide external advice and recommendations to EPA ORD. A subcommittee of EPA's BOSC was created in 2004 and charged with reviewing the design, relevance, progress, scientific leadership, and resources of the Endocrine Disruptor Research Program.<sup>13</sup> The subcommittee noted that the results of the program are providing EPA with the information needed to make scientifically-grounded decisions. The program has been productive, of high quality, and consists of an appropriate "combination of problem-driven and core research that has stood the test of time." The subcommittee was also impressed with the enthusiasm and commitment of the investigators conducting research for the program. The BOSC subcommittee noted that the continued success of the program will depend on many factors, including continued funding and interagency collaborations. Suggested areas for improvement included interactions with other agencies and addressing remaining research gaps.

"The EDC program is unique in that no other U.S. federal agency has such a program. The EDC program is not just an umbrella for a series of independent projects but is a fully integrated program across all ORD laboratories and centers (with the exception of the National Homeland Security Research Center)... The program is recognized nationally and internationally as a multidisciplinary set of research areas for both human health and wildlife and it cuts across the risk assessment/risk management paradigm."

— EPA BOSC, Subcommittee on EDCs,  
*Endocrine Disrupting Chemicals Research  
Program Review*, April 2005

In 2002, NAS independently reviewed the STAR grant program.<sup>14</sup> Using OMB guidelines for evaluating research programs, NAS evaluated the STAR program on quality, relevance, and overall performance. The NAS committee focused on three specific research programs, including endocrine disruptors. The review concluded that the program has played a vital role in funding exploratory and core research that would not otherwise be undertaken. The report observed that extramural funding through the STAR program was at the time one of the few federal programs

examining the impacts of endocrine disruptors on ecosystem processes. Others included USGS, NOAA, and the U.S. Fish and Wildlife Services.

**Quantity of Peer-reviewed Publications.** As of 2007, more than 500 peer-reviewed articles have been published representing a significant body of research.

#### ***Bibliometric Analysis of EDC Publications.***

To assess available literature, in 2007 program researchers conducted a bibliometric analysis of the more than 500 journal articles produced by the Endocrine Disruptors Research Program.<sup>15</sup> This analysis assessed the impact and influence of the published research using quantitative measures of citations to the articles and the popularity of journals where the articles were published.<sup>16</sup> The citation rates and journal impact factor data were calculated by ISI<sup>®</sup> Essential Science Indicators and Journal Citation Reports, respectively.

**Citation rates** indicate how frequently research articles are subsequently referred to in new publications. Average citation rates for a given subject field are calculated using the following ratio:

$$\frac{\text{Total citation counts of individual papers in the field}}{\text{Total number of papers published in the field}}$$

For example, the 2003 average citation rate in the field of chemistry was 1.31. This number means that the average publication in the field of chemistry that year has since been cited 1.31 times in other articles published in journals indexed by ISI. The results of the bibliometric analysis revealed the following statistics:

- The average EDC publication was cited in subsequent research articles about 17 times. The 519 total EDC publications were cited 8,997 times. The total number of citations was 8,579 when “self-citations” by the author were excluded.
- The EDC publications were more highly cited than the average. Citations of EDC publications in 11 of the 15 fields exceeded expected citations in those fields. This amounted to 489 of the 519 total publications (94%) analyzed.

“[STAR] research in endocrine disruptors ... has resulted in peer-reviewed groups of publications of immediate interest in understanding causes of, exposures to, and effects of environmental pollution.”

— NAS, *The Measure of STAR: Review of the U.S. Environmental Protection Agency’s Science to Achieve Results (STAR) Grants Program*, 2002

#### **EDC Papers Received STAA Awards**

EPA’s Science and Technological Achievement Awards (STAA) program implements an Agency-wide competition that recognizes outstanding science and technological papers (or series of articles) published by EPA staff. To date, intramural EDC researchers have won

- 1 Level I award (exceptionally high-quality research effort of national significance)
- 8 Level III awards (unusually notable research effort)
- 4 honorable mentions

The award program has recognized 26 EDC peer-reviewed articles.

U.S. EPA. 2007. Previous STAA Awards. Available at: [http://es.epa.gov/ncer/staa/annual/previous\\_awards.html](http://es.epa.gov/ncer/staa/annual/previous_awards.html).

- The authors of the EDC publications cited themselves less than the average self-citation rate. The self-citation rate of EDC publications, 4.6%, is well below the average range of 10 to 30% author self-citation rate.
- Seventeen of the authors of EDC publications are included in ISI Highly Cited.com. This is a database of the world's most influential researchers who have made key contributions to science and technology during the period from 1981 to 1999.
- Over 25% of the EDC publications are considered highly cited papers using the ESI criteria for the top 10% of highly cited publications. Of the 519 total publications, 14 are rated as highly cited using the ESI criteria for top 1% of highly cited papers. Four of these publications qualify as very highly cited when using the criteria for top 0.1%, and 3 papers meet the 0.01% threshold for extremely highly cited papers.
- Three of the EDC publications qualify as “hot papers.” Hot papers are papers that are cited shortly after publication. Papers must be cited within 2 years of publication and the citations must occur within a 2-month time period.

#### Very Highly Cited EDC Publications (i.e., Top 0.1%)

Field	Publications	Number of Cites
Environment/Ecology	Swan SH, et al. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. <i>Environmental Health Perspectives</i> 113(8):1056-1061.	71
Multidisciplinary	Zhu Y, et al. 2003. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progesterin receptor. <i>Proceedings of the National Academy of Sciences of the United States of America</i> 100(5):2237-2242.	181
	Zhu Y, et al. 2003. Cloning, expression, and characterization of a membrane progesterin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. <i>Proceedings of the National Academy of Sciences of the United States of America</i> 100(5):2231-2236.	182
	Anway MD, et al. 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. <i>Science</i> 308(5727):1466-1469.	105

## “Hot Papers” Identified Using ESI Thresholds<sup>a</sup>

Field	Paper	Number of Cites in 2-month Period
Environment/Ecology	Swan SH, et al. 2005. Decrease in anogenital distance among male infants with prenatal phthalate exposure. <i>Environmental Health Perspectives</i> 113(8):1056-1061.	12 cites in February-March 2006
	LeBlanc GA. 2007. Crustacean endocrine toxicology: a review. <i>Ecotoxicology</i> 16(1):61-81.	6 cites in February-March 2007
Multidisciplinary	Anway MD, et al. 2005. Epigenetic transgenerational actions of endocrine disruptors and mate fertility. <i>Science</i> 308(5727):1466-1469.	15 cites in May-June 2006

<sup>a</sup>ESI Thresholds are citation frequency thresholds specific to a publication’s field and a bimonthly period. Thresholds are set to retrieve 0.1% of the most highly cited papers within a 2-month period. For the current group of hot papers, the ESI threshold ranged from 4 to 9 citations.

**A journal’s impact factor** identifies the relative importance of a publication. It is determined by calculating the average number of citations for publications in a given journal over a 3-year period. For example, the 2006 impact factor for “journal A” would be calculated using the following ratio:

$$\frac{\text{Number of times publications in 2004 and 2005 are cited in indexed journals in 2006}}{\text{Total number of articles published in 2004 and 2005}}$$

A higher journal impact factor indicates that publications in that journal are cited more frequently.

- Nearly half of the EDC publications are published in very high-impact journals. Of the 519 papers analyzed, 213 appeared in the top 10% of journals, which represents 41.0% of the EDC publications.
- EDC publications in high-impact journals included 7 in the *Proceedings of the National Academy of Sciences of the United States of America*, with an impact factor of 9.493, 31 in *Environmental Health Perspectives*, which has an impact factor of 5.861, and 75 in *Toxicological Sciences*, with an impact factor of 3.598. This number is high relative to most journals; more than half of the journals evaluated by ISI have impact factors less than 1.<sup>17</sup>

**Use of Results in Hazard and Risk Assessments and Regulatory Decisions.** Program research has been used in EPA dose-response assessments. These assessments support risk assessments that inform EPA regulatory decisions, including setting safe tolerances for pesticide or for assessing risks from ingestion of industrial chemicals. Examples include:

- ORD research on the effects of vinclozolin on rat development and reproduction was used by EPA in the risk assessment that supported reregistration decision for vinclozolin. EPA used the data generated by ORD to establish the effects of concern and doses for the short- and intermediate-term non-dietary risk assessments and for characterizing remaining uncertainties.

- ORD's research on atrazine's pubertal effects, neuroendocrine disruption, and other adverse effects on fertility were used by EPA to develop a cumulative human health risk assessment of the chlorinated triazine class of pesticides.
- The University of Missouri-Columbia conducted EPA-funded research on the effects of phthalate levels in human mothers on male infant reproductive tract development. The study was cited in the determination of the oral reference dose (RfD) in the external peer-review draft of the Integrated Risk Information System (IRIS) summary for dibutyl phthalate.

## What impacts has the research had on the state-of-the-science?

By combining the resources of both intramural projects and extramural grants, the Endocrine Disruptor Program has made significant advances in addressing research gaps identified at the inception of the program.

Overall, the research program has successfully supported EPA's mission to protect human health and the environment and accomplished significant results in support of EPA goals to ensure clean and safe water and to protect, sustain, and restore the health of communities and ecosystems. ORD endocrine disruptor research has played a significant role in:

- ***Establishing a screening and testing program:*** Developed and validated methods for assessing endocrine disruptor activity with cell-based assays and with animal tests.
- ***Reducing uncertainty for risk assessment:*** Discovered mechanisms by which chemicals interact with mammalian and non-mammalian endocrine systems.
- ***Understanding dose-response relationships:*** Revealed that certain life stages are more sensitive than others to endocrine disruption.
- ***Provided data to support regulatory decision-making:*** Provided hazard and exposure data for EPA risk assessments.
- ***Provided predictive modeling tools:*** Developed tools to extend laboratory data to predict wildlife population-level effects and increase the relevance of test data for ecological risk assessments.

Specific research results on approaches for screening and testing (chapter 2), outcomes of early developmental and childhood exposure (chapter 3), and exposure and effects in wildlife (chapter 4) are described in the remaining chapters of this report. The following table presents some examples to illustrate the types of research results and their impacts.

## Where is the Endocrine Disruptors Research Program going in the future?

The Endocrine Disruptor Research Program will continue to address research gaps through the integrated intramural and extramural endocrine disruptor research program. EPA's program strikes a balance between "problem-driven" and "core" research. It includes areas that are uniquely of importance to EPA in helping it meet its legislative mandates and includes research areas that serve to improve the basic understanding of EDCs, in general, that is complementary

to research programs conducted at other federal agencies, in other countries, or by industry. ORD has significant expertise in the areas of toxicology, endocrine effects, behavioral sciences, and environmental exposures, relating to both humans and ecological systems and in providing solutions to solving environmental problems. ORD scientists are respected members of the scientific community and leaders in the field. Therefore, ORD can make a significant contribution in the areas of improving our understanding of endocrine disruptors, their impact on human health and the environment and the management of the risks they pose.

### Example Accomplishments in Major Research Focus Areas

Focus Area	Example Accomplishments and Impacts
<b>Screening and Testing</b>	
<p>Support the development and implementation of a screening and testing program for endocrine disruptor effects to meet statutory mandates (i.e., FQPA and SDWA Amendments).</p>	<p>Developed new and improved assays for use by the Agency's Endocrine Disruptor Screening Program (EDSP) and helped standardize, pre-validate, and validate protocols. ORD's research was essential to creating and refining 9 of 13 candidate Tier 1 assays.</p> <p>Developed a short-term and cost-effective Tier 1 amphibian metamorphosis assay that predicts thyroid hormone disruption.<sup>18</sup></p> <p>Contributed to the development and validation of Tier 2 tests for human health that include a mammalian multigeneration test and potential alternative approaches, mammalian in utero and lactational protocols.</p> <p>Developed test methods for bird and amphibian development and reproduction and a crustacean multigeneration test.</p>
<b>Early Developmental Exposure and Consequences</b>	
<p>Investigate underlying mechanisms of action for effects of EDCs from exposures during early development—a critical period of life when humans and other organisms may be particularly sensitive to EDCs.</p>	<p>Reported study results on atrazine and vinclozolin which provided critical information needed to improve the Agency's risk assessments and tolerance limitations for these pesticides.<sup>19,20</sup></p> <p>Determined concentrations of phytoestrogens in human amniotic fluid and evaluated effects of exposure to phytoestrogens in animal models.<sup>21</sup></p> <p>Determined that exposure to high concentrations of polybrominated biphenyls prenatally and in breast milk may affect puberty in girls.<sup>22</sup></p>
<b>Wildlife Exposures and Effects</b>	
<p>Improve understanding of the potential for adverse effects on individual organisms and populations of aquatic life and wildlife as a result of exposure to EDCs.</p>	<p>Identified feminization of male fish downstream from waste water treatment plants, masculinization of female fish downstream from paper mill effluent, and androgenic compounds in run-off from concentrated livestock farms.<sup>23,24,25</sup> EPA's Office of Water will consider this information as it develops water quality standards.</p> <p>Developed and transferred to EPA regional offices validated near-real-time DNA-based molecular indicator methods for characterizing estrogenic activity in environmental waters.<sup>26</sup></p> <p>Identified a new estrogen receptor in vertebrate fish (Atlantic croaker).<sup>27</sup></p>

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## CHAPTER 2. ADVANCES IN SCREENING AND TESTING

A variety of chemicals have been found to disrupt the endocrine systems of animals in laboratory studies, and compelling evidence shows that endocrine systems of certain fish and wildlife have been affected by chemical contaminants, resulting in developmental and reproductive problems.

Based on this and other evidence, the 1996 Food Quality Protection Act (FQPA) directed the U.S. Environmental Protection Agency (EPA) to develop a chemical screening and testing program using appropriate validated test systems to determine whether certain substances may have hormonal effects. In addition, Congress amended the Safe Drinking Water Act (SDWA) in 1996 and gave EPA the authority to test chemical substances in drinking water sources for hormonal effects if a substantial population may be exposed.

### Research Program Established to Support Regulatory Mandate

Before the 1996 amendments, few reliable endocrine disruption testing and screening tools existed. In 1998, EPA released a framework for an Endocrine Disruptors Screening Program (EDSP) based on recommendations from an advisory panel providing details of the approach for implementing the program. EDSP will initially focus on estrogenic, androgenic, and thyroid hormone effects.

These three hormone systems are presently the most studied of the known vertebrate hormones, and *in vitro* and *in vivo* test systems to examine effects exist and are amenable for regulatory use. Further, inclusion of estrogen, androgen, and thyroid

effects will cover aspects of reproduction, development, and growth. To provide the scientific basis for the mandated screening and testing program, EPA established the Endocrine Disruptors Research Program, one goal of which is to support EPA's EDSP.

EPA is currently developing and validating *in vitro* and *in vivo* assays to determine the potential for chemicals to cause endocrine disruption in humans or wildlife. EPA is using a two-tiered approach using a battery of assays. The purpose of the Tier 1 Screening is to identify substances that have the potential to interact with the estrogen, androgen, or thyroid hormone systems through any of several recognized modes of action. The purpose of Tier 2 Testing is to identify and establish a dose-response relationship for any adverse effects that might result from the interactions identified through the Tier 1 assays.

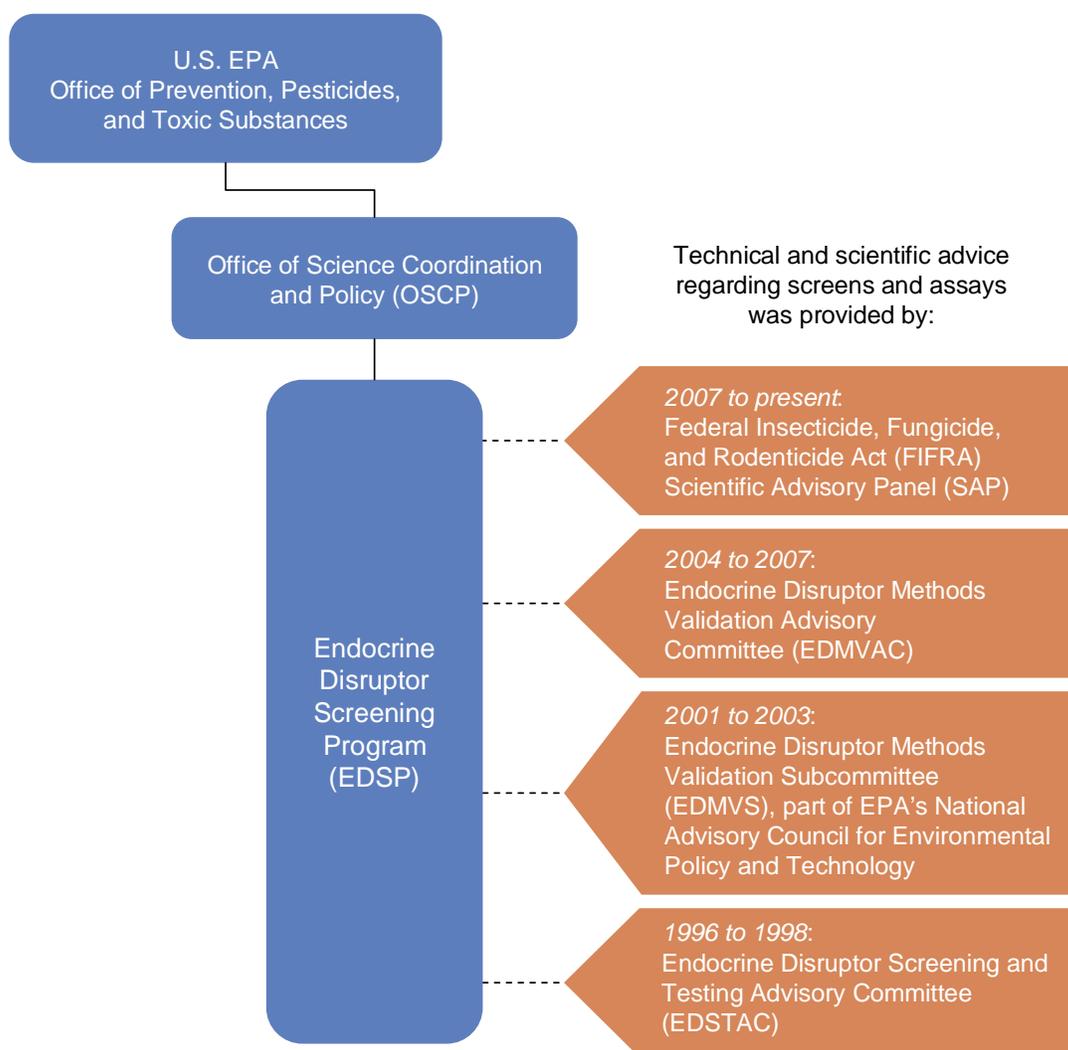
Long-term Goal 3: EPA's Office of Prevention, Pesticides, and Toxic Substances using endocrine disruptors screening and testing assays developed by ORD to create validated methods that evaluate the potential for chemicals to cause endocrine-mediated effects in order to reduce or prevent risks to humans and wildlife from exposure to endocrine disrupting chemicals

— ORD *Multi-Year Plan for Endocrine Disruptors*

"Each assay and test recommended for [Tier 1 Screening] or [Tier 2 Testing] needs some level of validation, standardization, methods development, or further research before being accepted as a regulatory toxicity screen or test for inclusion in the [EDSP]."

— EDSTAC Report, 1998, page ES-15

## Organization and Advisory Input for the Endocrine Disruptors Screening and Testing Program



### EPA Research Advances Screening and Testing Program

Research conducted by EPA's Endocrine Disruptors Research Program has resulted in validated tools and assays to screen chemicals for their potential to affect the endocrine systems of humans and wildlife. The program has provided the technical expertise to develop and improve approaches for identifying EDCs and their effects while also reducing the need for animals in the screening and testing program.

The screening tools and assays fall into three broad categories:

- **Priority-setting tools** assist in the selection of chemicals for endocrine disruptor screening and testing.
- **Tier 1 screening assays** identify chemicals with a potential to interact with the endocrine system.

- **Tier 2 testing assays** determine specific effects caused by each endocrine disruptor and establish the dose at which the effect occurs.

Screening and testing methods must undergo a five-step process to become validated and readied for implementation. The validation process is detailed in the accompanying figure along with EPA Office of Research and Development (ORD) contributions throughout the process. The EPA screening and testing methods under consideration are at various stages of development, standardization, and validation. Tier 1 screening assays are near completion and will be implemented starting in 2008.<sup>1</sup>

### ***Prioritization for Screening and Testing***

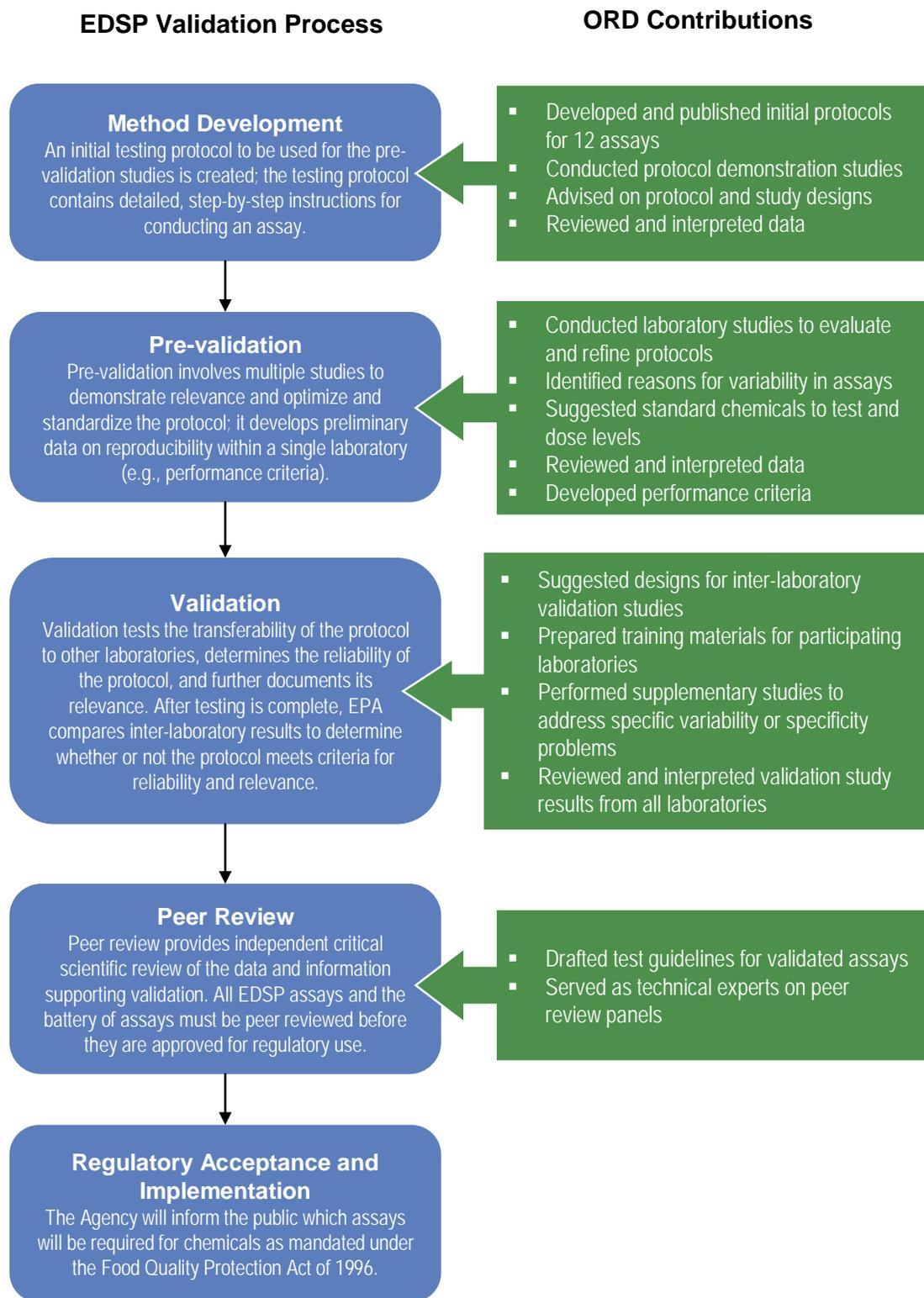
More than 87,000 chemical substances including active ingredients in pesticides, commercial chemicals, nutritional supplements, food additives, and environmental contaminants are to be characterized for endocrine disrupting potential. EPA needs to focus limited testing resources on chemicals with the highest potential for endocrine disruption and has established a priority-setting approach for choosing chemicals for screening. In the absence of other relevant data, EPA is considering legal mandates and the likelihood of exposure in the first selection of chemicals for screening evaluation.<sup>2</sup>

Quantitative structure-activity relationship (QSAR) is a process used to quantitatively correlate chemical structure with a well-defined process such as biological activity or chemical reactivity. One limitation of traditional QSAR is that it assumes a chemical has a single structure that is evaluated for its binding affinity with a hormone receptor. Using the hypothesis that a chemical can have more than one structure or conformation in complex environments such as biological tissues, which may have implications for interactions with hormone receptors,<sup>3</sup> ORD investigators developed a new model for predicting a chemical's binding affinity to a human estrogen receptor.<sup>4</sup> The model was applied to 46 chemicals with known and measured estrogen receptor binding affinities to predict binding affinities. EPA investigators successfully matched three-dimensional chemical structural characteristics of untested chemicals with those of known endocrine disruptors to predict the estrogenicity of alkylphenols.<sup>5</sup> This research showed that the binding affinities of specific chemicals to specific endocrine receptors can be modeled using this approach.

Research on prioritization tools has also helped identify chemicals capable of binding to the androgen receptor.<sup>6</sup> Structural features and binding interactions of several chemicals with structures similar to organophosphorus pesticides were identified and correlated with the ability to interact with androgen receptors. Consideration of a chemical's flexibility characterized by the distance between charged areas on the molecule is a critical parameter for the assay to detect potential binding. The study results are important for future attempts to construct models for predicting potential androgen activity.

More recently, research at EPA is actively investigating computational approaches for developing efficient strategies for prioritizing chemicals for screening and testing. Congressional action in fiscal year 2002 directed EPA to explore the use of alternative methods to animal testing for hazard assessment. ORD interpreted this as an opportunity to evaluate genomic and

## Endocrine Disruptors Research Program Contributions to the EDSP Validation Process



computational tools for screening purposes, and it initiated several research projects on EDCs as a “proof-of-concept” effort. Endocrine disruptors were selected because it was felt that a considerable amount of knowledge concerning mechanisms of actions and toxicity pathways existed for this class of environmental pollutants. The effort in this proof-of-concept activity is directed at developing better tools and assays to monitor selected aspects of endocrine disruption. Success will be measured against the recommendations put forth by EDSTAC and will provide confidence that the approaches are applicable to other pathways of toxicity where the underlying biology is not so well understood at the present time. The activities include refining existing QSAR models, developing *in vitro* models, and characterizing toxicity pathways.

An important component of EPA’s computational toxicology program relevant to EDCs is the ToxCast™ research program. Directed by ORD’s National Center for Computational Toxicology, the program’s goal is to develop cost-effective innovative approaches to quickly prioritize a large number of chemicals for toxicity testing. ToxCast™ is building computational tools that will help forecast the potential human toxicity of chemicals, including endocrine disruption. With sufficient information gathered from state-of-the-art high throughput screening bioassays, EPA believes it can identify distinctive patterns of results that are strongly correlated with specific types of toxic effects observed in animal toxicity tests. ToxCast™ is still in the research and development phase so it is not suitable for priority setting for the EDSP at this time. When sufficient data have been generated and analyzed, EPA will determine how best to use ToxCast™ for the EDSP.

### ***Tier 1 Screening Assay Accomplishments***

Tier 1 *in vitro* and *in vivo* screening assays are conservatively designed to detect endocrine disruptors. The screening approaches aim to minimize the number of false negatives at this stage of testing; in other words, the screening tests may identify chemicals that interact with estrogen, androgen, and thyroid hormones.

The endocrine disruptor Tier 1 screening assays and ORD contributions are listed in the table that follows. ORD’s research is essential to 9 of 13 candidate Tier 1 assays.

ORD research has advanced the science for Tier 1 laboratory screening tests. The following sections describe some examples of ORD projects that use cell line development for receptor binding assays and species-crossing evaluation of binding assays.

### ***Development of Cell Lines for Androgen and Estrogen Receptor Binding Assays***

ORD investigated alternative assays for the traditional rat estrogen receptor binding assay, which has the following drawbacks: (1) It requires the use of radioactivity and animal testing, (2) it is not amenable to high throughput testing, and (3) it does not determine if an EDC stimulates or inhibits the hormone receptor. One approach ORD investigators have taken is to genetically engineer cell lines to respond when cells are exposed to hormonally active test chemicals. Stable cell lines can improve the reproducibility and reliability of the assay for EDC screening. ORD developed a new cell line for evaluating estrogen receptor response. By cultivating breast cancer cells with specific estrogen receptors, the researchers created a stable cell line.<sup>7</sup> The project results support a specific, sensitive estrogen-responsive gene expression assay that is adaptable for high-throughput screening of large numbers of chemicals for estrogenic and anti-estrogenic activity. A stable cell line useful in identifying compounds that bind to the androgen receptor has also been developed.<sup>8</sup>

### Tier 1 *In Vitro* Screening Assays and Research Contributions

Assay	Description	Research Contributions
<b>Rat cytosol androgen receptor binding</b>	Uses rat prostate cytosol to examine a test chemical's ability to bind with androgen receptors, which are involved in male sexual characteristics development	ORD played a major role in development and validation
<b>Human and rat recombinant androgen receptor binding</b>	Uses a human and rat recombinant androgen receptor to detect the ability of a test chemical to bind with androgen receptors	ORD developed new cell lines and methods as alternatives to the rat cytosol approach, which may reduce animal use, and EPA scientists are collaborating with Japan and Europe on pre-validation studies
<b>Rat cytosol estrogen receptor binding</b>	Uses rat uterine cytosol to examine a test chemical's ability to bind with estrogen receptors, which are involved in female maturation and reproductive function	ORD characterized the estrogen receptor binding affinities of 50 industrial chemicals, <sup>9</sup> which will be part of the validation package for the estrogen receptor assay
<b>Human recombinant estrogen receptor binding</b>	Uses the alpha isoform of the human recombinant estrogen receptor to detect a test chemical's ability to bind with estrogen receptors, which is advantageous in eliminating the need for animal tissue	ORD produced novel cell lines and methods, and EPA scientists are collaborating with Japan and Europe on pre-validation studies
<b>Aromatase</b>	Focuses on the steroidogenic pathway to detect substances that inhibit aromatase enzyme activity; aromatase enzyme converts androgens into estrogens, estradiol, and estrone in all vertebrates	ORD supported improvement of this protocol, recommended alternate enzyme sources, improved technical assay aspects, provided solutions to technical problems, and reviewed data from contract laboratories
<b>H295R cell line steroidogenesis</b>	Detects interference with the body's production of male and female steroid sex hormones by using the H295R human adrenocortical carcinoma cell line, which can detect chemicals that can induce or inhibit the activity of enzymes responsible for steroid synthesis	ORD explored using the H295R cell line as a possible screening method. <sup>10,11,12</sup> EDMVAC recommended that this cell-based assay undergo pre-validation studies and identified it as a superior alternative to the sliced-testis steroidogenesis assay that uses animal tissues and had limited ability to discern specific endocrine toxicity from overt cell toxicity <sup>13</sup>

ORD genetically engineered a new cell line from breast cancer cells using an adenovirus to insert an enzyme-producing gene that glows when a nearby gene of concern is present. This reporter gene enables quantification of androgen receptor response.<sup>14</sup> The Expert Panel of the Interagency Coordinating Committee on the Validation of Alternative Methods recognized the advantages of the method, concluding that the adenovirus infection method provides a most promising avenue for assessing androgen receptor activity. This assay is beneficial because it is more straightforward and takes less time compared to previous procedures.<sup>15</sup> Although the Expert Panel did not in 2003 recommend a specific androgen receptor laboratory screening method as a

priority for validation, it suggested that protocols such as the one ORD developed would be the most effective and reliable for screening.

### ***Evaluation of the Accuracy of Receptor-Binding Assays across Species***

An EPA Science to Achieve Results (STAR) grant to Michigan State University investigated estrogen receptor-mediated activity of a structurally diverse set of compounds using laboratory and clinical testing assays for different species. The investigators discovered that while the function of estrogen and estrogen receptors was conserved between species, the amino acid sequences of the specific functional areas (or domains) of the receptors were less conserved, suggesting that using a single test species is not sufficient to screen and assess risks of estrogen disruptors.<sup>16,17</sup> Investigators used 61 mixtures at various concentrations, and later investigations tested 35 structurally diverse synthetic and natural estrogenic compounds (e.g., steroids, pharmaceuticals, phytoestrogens, mycotoxins, organochlorine compounds, polyaromatic hydrocarbons, and other industrial chemicals). Bacteria were genetically engineered to produce hybrid proteins that included three domains of an estrogen receptor. These hybrid proteins were then tested with estrogen receptors from five different species: humans, mice, chickens, green anole lizards, and rainbow trout. Competitive receptor binding identified several compounds that exhibited different interactions with estrogen receptor hybrid proteins. Qualitatively, all of the estrogen receptor proteins bound with the same set of compounds; however, there were substantial differences in relative binding affinities between species. For example, binding of chemicals with the trout estrogen receptor protein was temperature dependent and functioned more effectively near to physiological temperatures.<sup>18</sup> The data indicate that receptor binding screens are useful for prioritization of chemicals for testing but may not directly predict effects in whole animals.

### **Applications for Genomics and Bioinformatics Techniques**

EPA has applied genomics and bioinformatics techniques to understand the molecular basis of the toxicity of endocrine disruptors. Genomics is the study of DNA sequences and gene expression, while bioinformatics uses computer modeling and analysis to predict and explain biological functions and changes at the molecular level.

STAR researchers at Michigan State University studied gene expression profiles and adverse effects in mice exposed to several estrogenic compounds. An automated and customizable program to correct, filter, and normalize raw data from a commonly used gene expression microarray image analysis application (GenePix) was developed.<sup>a</sup> The results of the project included a review of bioinformatics approaches and examples for toxicologists to consider as tools for screening chemicals for interaction with the endocrine system and studying mechanisms of action.<sup>b</sup>

<sup>a</sup> Fielden MR, Halgren RG, Dere E, Zacharewski TR. 2002. GP3: GenePix post-processing script for the automated analysis of raw microarray image output files. *Bioinformatics* 18(5):771-773.

<sup>b</sup> Fielden, MR, Fertuck, KC, Matthews, JB, Halgren, R, Zacharewski T. 2002. *In silico* approaches to mechanistic and predictive toxicology: an introduction to bioinformatics for toxicologists. *Crit Rev Toxicol* 32(2):67-112.

### ***Male and Female Pubertal Assays for Estrogenic and Thyroid Disruption***

The Endocrine Disruptor Research Program projects explored *in vivo* screening assays for male and female pubertal protocols. The pubertal assays screen chemicals for sex hormone and thyroid activity during sexual maturation by exposing rats to the test chemical and examining them for abnormalities associated with sex organ development, changes in indicators of the onset of puberty, and thyroid tissue effects. Accomplishments include the following:

- ORD wrote two detailed review documents describing technical protocol issues and reviewed toxicology literature on pubertal alterations.<sup>19,20</sup>
- ORD investigated the suitability of the pubertal assay protocol by applying it to a pesticide, atrazine, known to alter secretion of luteinizing hormone and prolactin. The researchers sought proof using the assay to demonstrate that atrazine alters the development of male and female rat reproductive systems and the animals' thyroid function. ORD scientists administered atrazine by mouth daily to male and female rats for 20 and 9 days before\* the onset of the animals' pubertal development phase.<sup>21,22</sup> Atrazine significantly reduced ventral prostate weight in males and delayed vaginal opening in female rats. The assay demonstrated that the atrazine compound affected rat reproductive systems and thyroid function.
- ORD addressed concerns that the pubertal assays may inappropriately yield positive results simply based on body-weight changes. By conducting a restricted-feeding study, researchers showed that no interference is caused by body-weight loss as long as the difference is no greater than 10 percent of the control values. EDMVS used these results to set the maximum tolerated dose level for the pubertal screens.<sup>23</sup>
- ORD demonstrated that the Tier 1 male pubertal assay can be used to assess thyroid toxicity by various mechanisms. The results supported including pubertal assays in Tier 1 screening.
- ORD reviewed and analyzed data from an inter-laboratory study conducted for the validation process. ORD presented the analysis to OSCP, EDMVAC, and the public in April 2006.
- ORD applied the male pubertal assay to a fungicide, prochloraz, that inhibits steroid hormone synthesis.<sup>24</sup> Data from the ORD study along with other studies indicated that the sensitivity of the male pubertal assay could be significantly enhanced by measuring serum testosterone levels and testis hormone production. Improved assay sensitivity would reduce the number of animals required and lower assay cost.

### ***Hershberger Assay for Androgen Disruption***

The Hershberger assay detects androgenic and anti-androgenic effects measuring accessory sex gland weights in castrated adult or immature male rats. The Hershberger assay also provides critical information on the mechanism of a suspected EDC, which is important information for risk assessments of individual chemicals and mixtures. ORD was instrumental in validating this assay, by assisting with optimization and standardization and determining sensitivity, specificity, and reproducibility in an inter-laboratory validation study.<sup>25</sup>

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\* Males dosed postnatal day 23 to 53; Females dosed postnatal day 22 through 41.

ORD scientists assisted in the selection of chemicals for an inter-laboratory comparison and helped train laboratory personnel on operating procedures. As part of the project, ORD developed a guidance manual to standardize the dissection procedures for the male sex accessory tissues of interest. The manual was published with the protocol and included detailed photographs illustrating the step-by-step dissection of each tissue. ORD also helped analyze the validation study data and reported the results to OECD. ORD published results of Phase 1 of this research in peer-reviewed literature. This phase is intended to identify *in vivo* activity of suspected androgens and anti-androgens by testing the reproducibility and sensitivity of responses of male sex accessory tissues and other biological changes to androgen at varying doses. Seventeen laboratories from seven countries participated in phase 1 and the results indicated that the OECD Hershberger protocol was robust, reproducible, and transferable across laboratories. The inter-laboratory study demonstrated that results of this assay are highly reproducible with both potent and weak androgenic or anti-androgenic compounds. ORD scientists will continue to serve as technical experts for the OECD peer review of the test guideline, which is planned for 2007, while also offering assistance when OECD implements the next testing phase in 2007.

“The progress on Long-Term Goal 3 [support the Screening and Testing Program] within the Endocrine Disruptor Research Program has been excellent.”

— EPA BOSC Subcommittee on Endocrine Disrupting Chemicals, *Endocrine Disrupting Chemicals (EDC) Research Program Review*, April 2005

#### ***Amphibian (Frog) Metamorphosis Assay for Thyroid Hormone Disruption***

Frog metamorphosis is governed by the thyroid gland and is an excellent vertebrate model for assessing disruption of the thyroid hormone axis. The amphibian metamorphosis assay uses tadpoles to determine if chemicals affect the thyroid during metamorphosis and consequently result in developmental effects. ORD provided support for the development and pre-validation of the protocol. EDSTAC had recommended the use of a particular protocol that used the frog tail resorption as a measure of effect. However, there was concern that this proposed endpoint was not sufficiently sensitive to thyroid disruption. ORD scientists evaluated the protocol and concluded that the tail resorption measure was not ideal because this metamorphic stage was too insensitive. The laboratory examined an earlier prometamorphosis stage, when the larvae become responsive to the thyroid hormone and production of the thyroid hormone is initiated. From this research, ORD investigators developed a 14-day version of the prometamorphosis protocol with African clawed frog (*Xenopus laevis*) for the amphibian metamorphosis assay, and it was found to be sensitive to perchlorate, a thyroid disrupting chemical.<sup>26</sup> This protocol and pre-validation data were presented to OECD who considered it as well as a German 21-day amphibian metamorphosis protocol. A 14-day assay is more practical for the EDSP screening battery. However, the 21-day assay is initiated earlier in development, when frog larvae are more responsive to thyroid hormone, and it allows for a longer exposure time. In an international collaboration, three laboratories, one in Germany, one in Japan, and the other in the United States, tested both proposed protocols with two chemicals and found that, despite slight differences in study design, they were generally comparable. OECD recommended proceeding with validation studies using the 21-day protocol.

ORD scientists are continuing to investigate mechanisms of thyroid hormone disruption in the African clawed frog that may lead to shorter, less expensive assays with improved specificity.

They supported the refinement of the 21-day screening assay protocol that is now in the final stages of validation. The focus of this assay is organism development and thyroid gland histology, and ORD established a time course for histological changes in response to model thyroid hormone synthesis inhibitors. ORD, OECD, and OSCP cooperatively developed draft guidance document for preparation and microscopic analysis of thyroid gland tissue cells.

### ***Fish Screen Assay for the Detection of EDCs***

Included in the EDSP screening battery is a fish screening assay to identify endocrine and androgen-active chemicals for these animals. EDSTAC had proposed the fish gonadal recrudescence protocol for this purpose. ORD scientists evaluated whether this protocol was suitable as a fish screening assay, and the results of their studies suggested that this protocol was not effective due to high response variability among the fish. ORD investigated alternative measures of estrogen or androgen disruption and developed a short-term 21-day screening protocol using fathead minnow (*Pimephales promelas*).<sup>27</sup> This fish testing protocol analyzes estrogenic and androgenic activity of test chemicals by examining survival, reproductive behavior, secondary sex characteristics, histopathology, fertility, and development of offspring. They evaluated the protocol by testing methoxychlor and methyltestosterone, representative estrogen and androgen receptor agonists.<sup>28</sup> Both chemicals reduced fecundity and altered some endocrine pathways. A variation of the assay was also developed where fathead minnows were injected rather than exposed through the ambient water.<sup>29</sup> The injection method of dosing allows testing of chemicals that would require a large volume of test chemical to achieve the desired concentration in ambient water.

Input from EDMVS leaned towards general acceptance of the full 21-day version of the short term fish reproduction assay over a shorter 14-day version. The full 21-day version protocol was initially validated by an ORD laboratory. OECD then examined the fish assay with the cooperation of the United States and other countries.

### ***Tier 2 Testing Assay Accomplishments***

Tier 2 testing assays will be used to evaluate chemicals that are found positive as potential endocrine disruptors when screened in the Tier I screening battery. Chemicals selected for testing in Tier 2 are selected based on their relatively high potential for human exposure, rather than using a combination of exposure-and effects-related factors. EPA will focus on pesticide chemicals, already mandated for testing by Congress, followed by commercial chemicals, cosmetic ingredients, food additives, and nutritional supplements. The 50 to 100 chemicals selected for screening under the Food Quality Protection Act will undergo Tier 1 Screening first to identify their potential to interact with estrogen, androgen, and thyroid hormone systems. Chemicals found to be positive in Tier 1 will be tested in Tier 2. The Tier 2 assays will identify adverse effects caused by EDCs and establish a dose-response relationship for assessing hazard levels. Tier 2 tests are still undergoing validation and selection, but will likely include assays that (1) evaluate sensitive developmental life stages, (2) identify specific hazards caused by the chemicals and establish a dose-response relationship, and (3) cover a range of taxa.

The Endocrine Disruptor Research Program contributed to the development and validation of Tier 2 tests for human health including a mammalian multigeneration test and a potential alternative test to the standard mammalian *in utero* and lactational protocols. To assess ecological effects of EDCs, tests for bird and amphibian development and reproduction and a crustacean multigeneration test are being developed.

### ***Mammalian Multigeneration (Two-Generation) Assay***

ORD is finalizing a revised multigeneration development and reproductive protocol to evaluate reproductive toxicants to better characterize the dose response curve and define the range of adverse outcomes that result from exposure during gestation, lactation, and adulthood. Research team members have worked closely with the Office of Prevention, Pesticides, and Toxic Substances (OPPTS) to develop an alternative test protocol that would serve both the Endocrine Disruptors Screening and Testing program and the requirements for registration of all new pesticides and food additives. ORD recently proposed this Lifestages assay to the International Life Sciences Institute Lifestages Task Force.<sup>30</sup> This new protocol is part of a broader set of modifications proposed for assessing the safety of chemicals during the registration process. Among other things, the new Lifestages protocol has the primary advantage over the current test protocol because it will require fewer rats for use in the testing.

### ***Mammalian In Utero to Lactational Assay***

In recommendations to improve Tier 2 assays for testing androgens and anti-androgens, ORD recommended an approach to reduce animal use and enhance statistical detection of low incidences of adverse effects.<sup>31</sup> ORD is determining whether an alternative protocol can provide test results with less time and fewer animals than the existing test method to assess reproductive and developmental effects across generations. The proposed alternative assay involves administering the chemical to pregnant and lactating rats and then assessing the development of newborn pups after their exposures within the womb and through breast milk. ORD tested 21 pesticides, phthalates, dioxins, polychlorinated biphenyls (PCBs), and steroids using prototype versions of the assay. These studies provide a framework for assessing the usefulness of the assay. The assay is more sensitive in the low-dose region compared to the standard test, even though it uses fewer total animals and is shorter in duration than the standard test. ORD researchers improved the accuracy of the method by identifying sensitive measures for assessment according to each chemical's pharmacological activity in the screening battery.

### ***Frog Development and Reproduction Tests***

This project specifically addresses the development and standardization needs associated with an amphibian growth and reproduction full life cycle test. A protocol for an amphibian growth and reproduction test was requested by OSCP. A variation of the protocol will also be proposed as a test method in support of an effort by OECD to develop assays for internationally-harmonized EDC testing. The testing method is being developed by ORD in collaboration with scientists from the EU and Japan.

The assay uses frogs (*Xenopus tropicalis*) to characterize dose-response characteristics and adverse reproductive and developmental effects. ORD scientists developed partial life-cycle testing methods for amphibian species that evaluate early embryonic development and metamorphosis.<sup>32,33,34</sup> Partial life-cycle testing for determining the effects of a chemical on reproductive potential begins before the animal reaches sexual maturity and ends after the first reproduction. No standardized tests are currently available for evaluating chronic effects on development and maturation of the amphibian hypothalamic-pituitary-gonadal axis.

### ***Aquatic Invertebrate Two-Generation Test***

EDSP proposed mysids (*Americanmysis bahia*), a shrimp-like crustacean, as a suitable invertebrate for reproductive and developmental toxicity testing for EDCs. Mysids, which occur

in most aquatic environments, are important to many freshwater, estuarine, and marine food webs.<sup>35</sup> Mysids have been cultured for laboratory testing, and ORD researchers developed a protocol for a two-generation test that considers reproductive fitness in parents and offspring and measures other characteristics to test for endocrine disruption potential.<sup>36</sup> The researchers tested the method with a pesticide, fenoxycarb, which is known to act as an EDC in other organisms.<sup>37</sup> Results indicated that unexposed juvenile mysids whose parents were exposed to fenoxycarb produced fewer offspring and fewer males among their young. These reproductive effects on the offspring occurred at levels below those that caused parent reproductive effects. This is an important finding because it shows a transgenerational effect: future generations with no exposure can exhibit reproductive effects as a result of their parents' exposure. These findings support the use of two-generation invertebrate studies as an appropriate screening procedure to evaluate the effects of potential EDCs in aquatic environments.

An EPA STAR grant to the University of South Carolina resulted in the development of a copepod (*Amphiascus tenuiremis*) life-cycle microplate screening assay to evaluate reproductive, survival, and developmental effects on these marine and estuarine crustaceans.<sup>38</sup> The assay is being validated by OECD as a Tier 2 test for EDCs.

## **Exploratory Research into Alternative Laboratory Models**

Some early developmental pathways are remarkably conserved across species, providing opportunities to develop models of disease or early development stages that facilitate research to identify EDCs and elucidate mechanisms of endocrine disruption. EPA-sponsored endocrine disruptor research is continuing to research alternative species for testing, such as birds, and has also resulted in new cell-based or non-mammalian models. These projects include research on using a Japanese quail model for identifying EDC developmental effects on birds, a spiny dogfish shark model for evaluating effects on spermatogenesis, and a zebra fish embryo model to monitor gene expression in response to EDCs.

### ***Tests for Development and Reproduction Effects in Birds***

An EPA STAR grant to the University of Maryland investigated Japanese quail (*Coturnix japonica*) to develop a test for the effects of EDCs on birds and to identify reliable biological indicators of endocrine disruption. The Japanese quail is a promising model species because newly hatched birds are mature and mobile, the reproductive biology has been well characterized, and sexual maturity occurs only 8 weeks after hatching.

The University of Maryland researchers evaluated sublethal actions of selected EDCs (e.g., methoxychlor, vinclozolin, a polychlorinated biphenyl (PCB)) and the positive control estradiol benzoate in Japanese quail. They set out to determine (1) which phases in the life cycle (embryonic, maturing, or adult) are most vulnerable to insult by EDCs; (2) whether maternal exposures result in effects in offspring through transfer of EDCs or their metabolites into the eggs; and (3) which components of the endocrine and neuroendocrine systems (e.g., gonads, hypothalamus-pituitary) are primary targets of the EDCs and their mode of action. To answer these questions, the investigators conducted egg injection experiments, maternal transfer studies, and multi-generation tests.

Egg injection studies were used to assess the effects of estrogenic and anti-androgenic EDCs on embryonic development and subsequent sexual maturation and function of Japanese quail. These experiments revealed reduced fertility and egg production in estradiol benzoate-treated

pairs compared with both control groups. In particular, mounting behaviors of estradiol benzoate-treated males were dramatically reduced even though plasma androgen levels were unaffected. In estradiol benzoate-treated females, initiation of egg-laying was delayed 8 to 10 days and laying rate was reduced compared with controls (from 81 to 52 percent per day), although ovary weight was not affected. Adult males hatched from eggs treated with 25 or 50 ppm vinclozolin mounted females less often than control males, and the same result was found for adult males hatched from eggs treated with both doses of methoxychlor. For males from eggs injected with a PCB, the delay to mount was significantly increased compared with controls. Thus, all three EDCs interfered with the development of normal male mating behavior in ways consistent with their classification as weakly estrogenic or anti-androgenic. The results indicate that standard indicators of reproductive fitness and fertility are less sensitive than other measures such as behavior, sexual maturation, and gonadal morphology.<sup>39</sup>

In the maternal transfer studies, methoxychlor and the soy isoflavone, genistein, a phytoestrogen were administered orally in gel capsules to groups of hens daily for five days and then the eggs were examined. The results indicated that the compounds tested were readily maternally deposited and sequestered into the yolk.

The multi-generation experiments conducted with methoxychlor were administered in the diet of the quail to assess potential effects of

environmentally-relevant dietary exposure levels. Several of the measures of effect examined appear to be potentially reliable indices of low-level EDC exposure. Among these measures, plasma steroid hormones were reduced in both males and females of the parent, first generation, and second generation birds compared with controls. Birds exposed to higher concentrations of methoxychlor in their diet exhibited higher rate of reproductive failure (defined as lower viability of chicks and a higher proportion of birds that did not become reproductive), which was reflected in their lower plasma steroid hormone levels. Delayed maturation and lower plasma steroid hormones were accompanied by impaired sexual behavior in males and more variable responses related to fertility, egg production, and body weight in females. This variability was due in part to individual differences in response to the pesticide exposure, with some birds maturing at a normal rate and others showing delayed onset of mating behaviors or egg production. Neurochemical measures, including hypothalamic catecholamine concentrations and aromatase activity, revealed similar dose-related changes the parent and offspring for both generations, suggesting that these neuroendocrine modulators of reproduction are important targets of EDCs.

The studies suggested that embryos represent the most vulnerable life stage of the quail, while adults represent the least vulnerable. The results pinpointed several sensitive neuroendocrine, endocrine, and behavioral variables as endpoints for testing paradigms that were not part of



University of Maryland researchers concluded that Japanese quail provide a useful model for assessing endocrine disruptor effects on birds.

current EPA or OECD toxicity testing guidelines for avian species. The time required to reach sexual maturity for both males and females and the vigor of male mating behaviors were particularly sensitive indicators of EDC exposure. Egg injection and the two-generation dietary tests provided the most complete and reliable data for establishing endocrine disruption in the quail model. In addition, the two-generation dietary testing paradigm provided the opportunity to assess the effects of EDCs at various life cycle stages at environmentally relevant exposure concentrations. These data support the design and development of one- and two-generation test methods for birds.

### ***Spiny Dogfish Shark Model***

Boston University investigated the synthesis and effects of estrogen within the testes of the spiny dogfish shark.<sup>40</sup> The spiny dogfish shark (*Squalus acanthias*) is a simple animal model for the study of spermatogenesis. The organization of the testis of the dogfish shark is technically advantageous for stage-by-stage analysis of spermatogenesis in cell-based and whole animal tests because of the organizational simplicity of its testis relative to man, rodents, and other common laboratory animals. Using the dogfish shark, the researchers were able to test the direct effects of estradiol-17 $\beta$  on physiological processes in areas of the testis rich with total estrogen receptors. They observed that estradiol-17 $\beta$  has dose-dependent inhibitory effects on both mitosis and apoptosis in stem cell and some stages of spermatogenesis. These results support the hypothesis that estrogen is part of an intratesticular negative feedback loop where more differentiated cells in the testes regulate the development of less mature regions. A growth control mechanism like this could help explain the temporal, spatial, and quantitative order of succeeding stages of normal spermatogenesis in all vertebrates.

### ***Zebrafish Model***

Fish embryo models, such as the zebrafish, have been shown to be a useful complement to mammalian models because the internal structures can easily be observed as they develop. Duke University Medical Center developed methods to produce transgenic zebrafish embryos that glow when a developmental pathway is expressed.<sup>41</sup> The zebrafish embryo is “infected” with a reporter gene that codes for a fluorescent protein that can be observed when certain genes are expressed. The center applied this approach to investigate the expression of estrogen receptor  $\beta$  during the first 48 hours of embryonic development. This research supports the development of dependable and consistent technologies for producing models that show, in real time, when and where developmentally important genes are expressed. This method, in turn, could support studies that assess how EDCs affect developmental pathways.

## **Impacts and Outcomes of Research**

The Endocrine Disruptor Research Program continues to supply critical information to help EPA meet its Congressional mandate to develop and implement the Endocrine Disruptor Screening Program. A summary list of accomplishments follows.

### **Endocrine Disruptor Research Program Screening and Testing Achievements**

- Research on prioritization tools to help focus screening efforts and limited resources on chemicals predicted to have endocrine disrupting potential.
- Significantly advanced development and pre-validation of all but one of the Tier 1 screening assays. Validation studies are complete for three Tier 1 assays: Hershberger assay, the male pubertal development assay, and the female pubertal development assay. Pre-validation studies are complete for four other assays: two estrogen receptor binding assays, one androgen receptor binding assay, and the aromatase inhibition assay.
- Developed and prevalidated four Tier 2 assays: mammalian multigeneration, amphibian development and reproduction, mysid two-generation, and the rat in utero-lactational protocol. ORD scientists prepared detailed reviews for these assays and pre-validation work is in progress. Program studies and ORD staff expertise played a critical role in this progress.
- Developed alternative Tier 2 testing methods for birds, amphibians, and copepods.
- Developed new test methods that incorporate advances in molecular and cellular biology and capitalize on new computational tools. These new methods hold promise for reducing the use of animals in future testing. In addition, these methods likely will lead to improved efficiency and reduced costs associated with testing.
- Participated on national and international workgroups and committees to develop harmonized guidelines and methods for detecting EDCs.

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## CHAPTER 3. UNDERSTANDING OUTCOMES OF EARLY DEVELOPMENTAL AND CHILDHOOD EXPOSURE

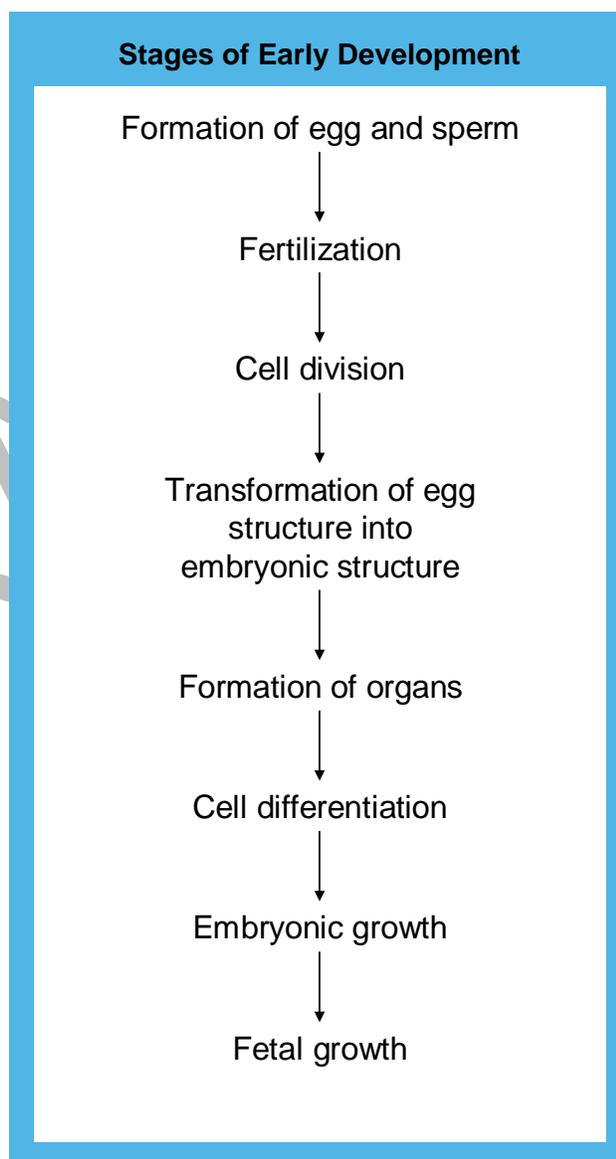
The biochemical and molecular processes of early development are subjects of intensive research to improve our understanding of what happens when incorrect events occur. Normal development of a single egg into an embryo, fetus, and functioning multi-cellular organism requires rapid and complex biological changes in a precisely timed sequence. The growth, differentiation, and maturation of structures and function are guided by intercellular signaling and changes in gene expression during early development and childhood. Hormones and other signaling molecules are the messengers of intercellular communication that guide early and childhood development.

Endocrine disrupting chemicals (EDCs) have the potential to disturb normal developmental processes by interfering with natural hormone systems. Adverse effects can arise when critical developmental events are altered by such EDCs, depending on the relative potency of the chemical, and the level, duration, and timing of exposure.

When the Endocrine Disruptors Research Program was initiated, early life stages were believed to be most sensitive to endocrine disruption, but the critical factors contributing to the sensitivity of developing organisms and how these factors influence health later in life was not known. Many hypotheses were driven by laboratory animal studies and by the tragic discovery that a synthetic estrogen (diethylstilbestrol) given to pregnant women from the 1940s to 1970s to prevent pregnancy loss led to reproductive tract malformations and cancer in their children. But mechanisms of endocrine disruption and dose levels associated with adverse outcomes—like many of the early developmental pathways—were largely unknown.

The objective of U.S. Environmental Protection Agency (EPA) research on early developmental and childhood exposure to EDCs is to determine the critical factors

contributing to the susceptibility of developing organisms to environmental endocrine disruptors and how these factors influence the health of adults. EPA Office of Research and Development (ORD) research focuses on new approaches to the study of developmental effects (including reproductive, immunological, and neurological). The results provide the ability to better



extrapolate to the human fetus and establish insight into how exposures lead to altered function and/or disease in adults. In specific cases, the results provide a better understanding of tissue concentrations of toxicants such as herbicides, anti-androgens, and selected disinfection byproducts known to alter endocrine function. ORD has provided detailed dose-response information for a variety of chemicals to EPA's program and regional offices and addressed important low dose issues, such as whether or not biological thresholds exist. The results of this research have already been used in setting tolerance levels for certain pesticides by the Office of Pesticides Program and for decision-making by the Office of Pollution Prevention and Toxics.

EPA research highlighted in this chapter determined relationships between endocrine disruptor exposure during critical developmental stages and adverse outcomes later in life. Research investigations examined effects on reproductive health (e.g., reproductive tract development, sexual maturation, and fertility); effects on the thyroid, nervous, and immune systems; and the development of cancer.

## **Effects on Reproductive Tract Development**

Endocrine Disruptor Research Program projects characterized mechanisms for abnormal reproductive development following exposures to EDCs. The research aimed to understand how—at the cellular and molecular level—EDC exposures cause adverse effects and provides data for EPA's risk assessments that estimate safe exposure levels for pesticides and toxic substances. These mechanistic data for EDCs are useful to (1) identify low dose effects, (2) characterize the shapes of the dose-response relationships, (3) identify critical measures of effect for risk assessments, and (4) reduce uncertainties in interpreting animal data for humans. The impacts of this research for human health risk assessment are illustrated with findings for anti-androgens vinclozolin and phthalate plasticizers and an estrogenic chemical, BPA.

### ***Vinclozolin Effects on Testosterone Levels Induce Reproductive Tract Malformations in Developing Rats***

Vinclozolin is a fungicide used to control disease on fruits, vegetables, turf grass, and ornamental plants in the early 1990s. ORD determined the effects of vinclozolin in rodents at different life stages (e.g., prenatal, postnatal, adolescence, and adulthood). They conducted short-, intermediate-, and long-term studies in which rats were exposed to vinclozolin in their diet.

ORD's research and other data were used by EPA in the risk assessment that supported the reregistration decision for vinclozolin.<sup>1</sup> EPA used the data generated by ORD to establish the effects of concern and doses for the short- and intermediate-term non-dietary risk assessments and for characterizing remaining uncertainties. The improved understanding of the mode of action of vinclozolin provided a more scientifically robust basis for extrapolating animal data to potential human response and provided information critical in identifying the point of departure for EPA's risk assessment of vinclozolin.

In their studies, ORD scientists exposed pregnant rats to vinclozolin during the period of their male offspring's growth and differentiation of sexual organs at doses as low as 3.125 milligrams per kilogram body weight per day (mg/kg/day).<sup>2</sup> At all dose levels, reproductive effects observed in the male offspring included significant reductions in anogenital distance (measured from the midpoint of the anus to lower base of the penis) and increased incidence of areolas. ORD scientists observed that although vinclozolin binds weakly to the androgen receptor, two metabolites of vinclozolin show anti-androgenic activity in cell-based tests.<sup>3</sup> The study suggested

that the demasculinizing effects of vinclozolin exposure may be mediated by the anti-androgenic metabolites.

ORD scientists also identified the period during the development of the male rat when it is most sensitive to the effects of vinclozolin.<sup>4</sup> The investigators orally dosed pregnant rats with vinclozolin, and each group received a dose on different 2-day consecutive intervals during gestation of the fetus. The results indicated that the reproductive system of the fetal male rat is most sensitive to vinclozolin's anti-androgenic effects on gestation day 16 and 17, which corresponded to the timing of the appearance of androgenic receptors in the male reproductive tract, particularly in the mesenchyme of the embryo that eventually develops into connective, circulatory, and other tissues. The investigators proposed that the reproductive structures are most susceptible to alteration by vinclozolin at the onset of structural differentiation, which coincides with the appearance of androgen receptors in the mesenchyme. Malformations can no longer be induced by the time androgen receptors are mainly located in the epithelium.

### ***Prenatal Exposure to Plasticizers Altered Male Reproductive Tract Development***

Phthalates are ubiquitous industrial chemicals used to make flexible vinyl for use in building and car products, clothing, food packaging, children's toys, and medical devices. They are among the most abundant synthetic chemicals in the environment.

ORD, EPA-funded researchers, and others determined that prenatal exposure to phthalates leads to incomplete formation of the male reproductive tract in male rats and rabbits. Pregnant rats orally dosed with dibutyl phthalate and diethylhexyl phthalate had the sexual differentiation of male offspring was altered in an anti-androgenic manner.<sup>5</sup> ORD examined six different phthalates and found that diethylhexyl phthalate, benzyl butyl phthalate, and diisononyl phthalate altered sexual differentiation of the male rat when their mothers were given 750 mg/kg during pregnancy.<sup>6</sup> There were significant incidences of reproductive malformations and the males displayed female-like areolas or nipples. The three other phthalates tested, diethyl phthalate, dimethyl phthalate, and dioctyl terephthalate did not induce these effects.

In another project, researchers at the Colorado State University studied the effects of dibutyl phthalate in rabbits. They selected rabbits because they have a relatively long infantile period of reproductive development, with spermatogenesis beginning in postnatal weeks 7 and 8 and mature spermatozoa appearing around weeks 13 to 14. This period of reproductive development—adolescence—in rabbits, relative to lifespan, exceeds that of rodents. Therefore, it better approximates the adolescence phase of human development. Rabbits also provide opportunities for multiple evaluations of endocrine profiles, mating ability, and semen quality.

Rabbits were orally dosed with dibutyl phthalate (400 mg/kg/day) during pregnancy at the time of fetal formation of the reproductive tract.<sup>7</sup> Similar to results observed for rats, the most pronounced reproductive effects were seen in male rabbits exposed in utero. The adult male rabbits exposed in utero showed a 43% reduction in numbers of ejaculated sperm and reductions in the weights of testes and accessory sex glands. Also observed were reduced testosterone levels and a doubling in the percentage of abnormal sperm compared to controls. The findings suggest that this dose level of dibutyl phthalate caused a permanent effect on the function of the male sex organs.

Driven by phthalate findings in animals, researchers at the University of Missouri-Columbia investigated phthalate levels in humans in relation to human male reproductive tract

development. The investigators assessed prenatal exposure of 85 infant boys to phthalates, as indicated by their mothers' prenatal urinary concentrations of phthalate metabolites, and evaluated the infants for growth and reproductive tract effects.<sup>8</sup> Physicians measured the infants' height, weight, head circumference, anogenital distance (measured from the midpoint of the anus to the lower base of the penis), and anoscrotal distance (measured from the midpoint of the anus to the anterior base of the penis) and examined the infants' bodies for breast and genital abnormalities.

Most women in the study had detectable urinary levels of the nine phthalate metabolites measured. No frank genital malformations or disease were detected among the assessed infants, but maternal urinary concentrations of four phthalate metabolites—monoethyl phthalate, mono-n-butyl phthalate, monobenzyl phthalate, and monoisobutyl phthalate—were inversely related to anogenital distance. That is, shortened anogenital distances were measured in infant boys whose mothers had elevated prenatal levels of these phthalate metabolites. Animal data for three of the metabolites suggest that rodents and humans show similar sensitivity. However, the metabolites of diethyl phthalate and monoethyl phthalate do not affect reproductive development in rodents. It is not known if this difference is because of differences in sensitivity between humans and rodents, unmeasured confounding factors in human studies, or limitations in animal studies. Follow-up research is needed to determine the persistence of the observed effects on anogenital distance and to identify long-term reproductive impacts, if any, of prenatal phthalate exposure in exposed children. The study was one of three human studies stated to be “useful” by the U.S. National Toxicology Program's Center for the Evaluation of Risks to Human Reproduction in its assessment of reproductive and developmental effects of di(2-ethylhexyl) phthalate.<sup>9</sup>

Additionally, the research on phthalates has been used for characterizing human health dose-response assessments that are used in chemical risk assessments.<sup>10</sup> For example, the ORD animal and human research on phthalates has been cited in the June 2006 peer review draft toxicological review of dibutyl phthalate. The University of Missouri-Columbia study was cited in the determination of the oral reference dose (RfD) in the external peer-review draft of the summary for dibutyl phthalate.

### ***Prostate Development in Rodents Sensitive to Estrogenic Chemicals***

Studies on rodents indicated that brief exposure to natural estrogens during early development results in permanent alterations of the prostate, including differentiation defects, abnormalities in cell maturation, cell proliferation, and benign growth formation with aging. Synthetic environmental contaminants with estrogenic properties might likewise affect the prostate gland. Exposure to estrogenic chemicals could be a basis for increased risk of prostate cancer among older men. Research sponsored the Endocrine Disruptor Research Program aimed to determine the consequences of exposure to EDCs, particularly environmental estrogens, during fetal prostate development. The research found that exposure estrogenic chemicals resulted in a permanent change in the structures of certain regions of the mouse prostate. Implications for human health include insights into effects of endocrine disrupting chemicals on human prostate development and prostate cancer risk associated with permanent changes in growth parameters in the urogenital tract and associated structures.

In the study, University of South Dakota investigators evaluated BPA, a chemical that is used in plastics and is released to the environment through oil and gas production and chemical, plastic, and electronic industry operations. Human exposure to BPA may occur from

## ORD Evaluated Prostate Cancer Risk Factors for Humans

Understanding the risk factors for cancer is important for protecting public health and developing effective policy. Prostate cancer poses a greater risk for American men, especially African-American men, than any other nonskin cancer. The higher prevalence of prostate cancer has resulted in part from successful efforts at early detection, with use of the serum prostate-specific antigen test. However, physicians are unable to diagnose patients accurately as to who will have progressive cancer and who will not. Another significant challenge is identifying which men are at greatest risk for developing prostate cancer.

In response to these challenges, ORD performed a comprehensive analysis of suspected risk factors including exposure to endocrine disruptors to the development of human prostate cancer. The results were presented at a conference in 2002 and published.<sup>a</sup> The paper identified several endogenous and exogenous risk factors for prostate cancer. Endogenous risk factors include family history, hormones (elevated levels of androgens), race (African-American men have the highest incidence of prostate cancer), and oxidative stress due to aging. Exogenous factors include diet (fat and retinoid consumption were found to increase prostate cancer risk, while vitamins D and E reduced it), environmental agents (EDCs may increase risk of cancer), and occupational hazards (some association between prostate cancer risk and work in the farm or rubber industries). The report states that more research is needed to explain the mechanisms of prostate cancer development and the possible carcinogenic effects of aging, mutagens in cooked foods, and androgens on prostate cells. The authors concluded that most of the data regarding risk relies, of necessity, on epidemiologic studies, but animal and cell culture models offer promise in confirming some important findings. The current understanding of biomarkers of disease and risk factors is limited.

<sup>a</sup>Bostwick DG, Burke HB, Djakiew D, Euling S, Ho SM, Landolph J, Morrison H, Sonawane B, Shifflett T, Waters DJ, and Timms B. 2004. Human prostate cancer risk factors. *Cancer* 101(10 Suppl):2371-2490.

dental sealants and ingestion of water or foods manufactured or packaged in BPA-based plastics. Using ethinylestradiol (EE), an active ingredient in oral contraceptives, as a positive control, pregnant mice were dosed daily with BPA or EE during male reproductive tract development on gestation days 14 to 18.<sup>11</sup> The BPA group received 10 µg/kg/day, and the EE group received a dose of 0.1 µg/kg/day. Just before normal birth, the investigators removed the male offspring and examined them for abnormalities of the prostate and urethra and used three-dimensional anatomical reconstructions to evaluate developmental patterns of these structures.

Low doses of BPA or EE produced an increase in the quantity and size of prostate ducts and an overall increase in prostate-duct volume in the male fetal mice. Urethras of the BPA-treated mice were significantly constricted where they enter the bladder, which could lead to urine-flow disorders.

These results, along with previously published data, demonstrate in a laboratory animal model that exposure to estrogens during critical periods of development in the male fetus caused alterations of growth parameters in the prostate which may persist into adulthood. The changes in the structure of the prostate and urethra induced by estrogenic chemicals may have implications for human disease. Low doses of BPA and EE caused structural changes in the region of the developing mouse prostate that is analogous to urethral ridges in humans that develop into the primary prostate secretory ducts. The majority of malignant prostate tumors form in the primary prostate secretory ducts in men as they age.

## **Effects on Growth and Sexual Maturation**

Development is a period when hormone-mediated changes in gene expression can have permanent consequences that may not be apparent until later in life because functional changes do not occur until puberty. Sex hormones and thyroid hormones are important in the regulation of growth and puberty in mammals. These complex processes—both the early developmental programming and the later in life pubertal changes—are potential targets of alteration by EDCs. Exposure to certain pharmaceutical and environmental compounds has been shown to alter the timing of pubertal development in animals. EPA decided that research was needed to characterize the extent of the impact of early developmental or childhood exposures to EDCs on growth and puberty. The Endocrine Disruptors Research Program investigated mechanisms of pubertal effects of EDCs on laboratory animals and funded epidemiologic studies of the growth and sexual maturation in children exposed to environmental EDCs. The body of research shows that changes in the timing of the onset of puberty are sensitive indicators of endocrine disruption.

### ***Estrogen Treatment Advanced Puberty Onset in Male Rats***

Researchers at the University of Illinois at Chicago investigated whether exposure to environmentally relevant doses of estrogenic chemicals during prostate development promotes abnormal maturation of cells or increase tumor formation with aging. However, their research revealed that low doses of an estrogen administered shortly after birth advanced the onset of the pubertal phase in male rats.

The researchers evaluated the effects of estrogen treatment on the prostate gland, male puberty, and the prostate for two strains of neonatal male rats.<sup>12,13</sup> When neonatal Sprague-Dawley rats were given (on days 1, 3, and 5 of life) a low dose of 0.15 µg/kg body weight of β-estradiol-3-benzoate, the investigators observed a temporary increase in relative prostate weights and supermasculinization of liver enzyme patterns (i.e., they showed increased expression of liver enzymes known to be under androgenic control). These findings were interpreted as being associated with advanced onset of puberty. In contrast, the high-dose groups (1,000 and 1,500 µg/kg) showed significantly decreased relative prostate weights and demasculinization in the liver activity patterns. This was the first observation that neonatal exposure to low doses of an estrogen can stimulate liver testosterone biotransformation enzyme activity in the prepubescent rat liver.

The results suggest that that the onset of the pubescent period is an important measure for estrogenic effects in males, and measures of liver testosterone biotransformation enzymes may serve as sensitive indicators of the onset of puberty.

### ***The Herbicide Atrazine Affected Pubertal Timing and Immune System Development in Rats***

Atrazine is a broad-spectrum triazine herbicide that was first registered with EPA for use in 1958. It is estimated to be the most heavily used herbicide in the United States, and its heaviest uses are on corn, sugarcane, and residential lawns. Studies to support its original registration indicated development of mammary tumors in a study of rats with chronic tumors and in a study of development of skeletal formation delays in rats. However, subsequent research by ORD characterized the dose-response for atrazine and its endocrine disruptor mode of action.

Research by ORD has demonstrated that atrazine delays the onset of puberty in male and female rats exposed before puberty.<sup>14,15,16,17</sup> More recently, ORD researchers evaluated the effects of

early developmental exposure and/or breast-milk exposure to atrazine on the timing of puberty in female and male rat offspring. The maternal rats were dosed orally with atrazine at a dose level of 100 mg/kg/day for 5 days during gestation when the fetuses' reproductive tissues were developing. All of the groups of female offspring that were exposed to atrazine—either gestationally, via lactation, or through both exposure routes—displayed significant delays in epithelial development in their mammary glands, and the findings did not appear to be related to body weight or endocrine hormone concentrations.<sup>18</sup> The offspring receiving only milk from atrazine-exposed mothers exhibited a significant delay in vaginal opening, an indicator of puberty in female rats. Males exposed to atrazine both in utero and via breast milk showed a significant 2.5-day delay in puberty, as measured by the timing of the separation of the foreskin of the penis from the glans<sup>19</sup> and significant reduction in body weight at puberty compared to the other groups.

ORD's research results indicating pubertal effects, neuroendocrine disruption, and other adverse effects on fertility were used in a cumulative human health risk assessment for EPA's reregistration decision for the chlorinated triazine class of pesticides.<sup>20</sup>

ORD scientists also evaluated the endocrine-mediated immunotoxicity of atrazine. They published the first study demonstrating that developmental exposure to atrazine causes gender-dependent immunosuppression of cellular and humoral function.<sup>21</sup> Pregnant rats exposed to 35 mg atrazine/kg/day from gestational day 10 through postnatal day 23 had a decrease in primary antibody and delayed-type hypersensitivity in male offspring. These results suggest that atrazine is a developmental immunotoxicant, but further studies are needed.

### ***Pesticide Methoxychlor Effects on Non-human Primate Growth and Development***

Monkeys, like humans, have a relatively long period of maturation during adolescence, and additional physiological similarities that make them useful models for understanding effects of estrogenic compounds during human adolescence. University of California at Davis researchers investigated the effects of the pesticide methoxychlor and the well-known estrogen agonist, diethylstilbestrol, on adolescent development in rhesus monkeys. This project aimed to provide information on adolescents as a sensitive population for endocrine disruption resulting in reduced fertility in adulthood, impairment in function of late maturing brain areas, enhanced susceptibility to pathogens encountered by young adults, and postmenopausal osteoporosis. The rhesus monkey model approximates the prolonged and complex adolescent period of humans. The research will help determine whether methoxychlor generally alters the onset and course of adolescent maturation through actions at hypothalamic regulatory sites, or acts more directly and discretely on tissues that mature under the influence of estrogen such as bone, brain, and immune systems, as well as the reproductive system.

In the study, methoxychlor (25 or 50 mg/kg/day) or diethylstilbestrol (0.5 mg/kg/day) was administered to female rhesus monkeys for 6 months before and after the anticipated age of initial menses.<sup>22</sup> Both methoxychlor and diethylstilbestrol led to premature emergence of a secondary sex characteristic, reddening and swelling of genital skin, but retarded nipple growth. As evaluated by ultrasound, uterine size was not affected, but there were indications of increased incidence of ovarian cysts/masses in the treated groups. Methoxychlor-treated monkeys showed altered ovarian cyclicity (shorter follicular stages) as monitored using urinary hormone metabolites. These data confirm that diethylstilbestrol has a major effect on adolescent

maturation in rhesus monkeys, and that the pesticide methoxychlor also altered development during this period.

Visual discrimination performance measured during dosing demonstrated delayed improvement and poorer performance in the high-dose methoxychlor and diethylstilbestrol groups. Visual recognition memory, assessed with delays of less than three seconds, was not apparently affected. Spatial working memory, assessed after dosing, showed acquisition deficits and possible working memory difficulties in the high-dose methoxychlor group. Late peak latencies of the auditory brainstem response were shorter in the diethylstilbestrol group 6 months after treatment, suggesting long-term effects on the brain. The study suggests that some aspects of brain function can be modified by exposure to exogenous estrogen during pubertal development. Although diethylstilbestrol is a more potent estrogen, the high-dose methoxychlor group was more affected behaviorally.

The results demonstrate that exposure to an estrogenic chemical during puberty can alter measures of reproductive development and functioning, memory, and learning in a nonhuman primate model. The results for a primate model provides data that reduces uncertainties associated with extrapolating laboratory animal data for estrogenic chemical human health risk assessment. The study also provides insights into the potential human health implications of early exposures to EDCs for adolescent girls.

### ***Growth and Puberty in Girls Exposed to Polybrominated Biphenyls (PBBs) During Early Development***

PBBs were used as fire retardants for consumer products including electronic equipment before being removed from the market due to toxicological concerns—test data suggested that long-term exposure to low levels of PBBs could induce weight loss, skin disorders, nervous and immune system effects, and effects on the liver, kidneys, and thyroid gland. In the early 1970s, PBB flame retardants were mistakenly mixed with cattle feed instead of a nutritional supplement manufactured at the same facility, exposing several thousand Michigan farm families and residents who consumed contaminated beef and dairy products. Because PBBs are structurally similar to polychlorinated biphenyls (PCBs), industrial chemicals with endocrine disruptor properties, Emory University researchers examined the occurrence of reproductive health outcomes related to endocrine disruption in PBB-exposed women and their children. This major epidemiologic investigation was funded by EPA, the National Institute of Environmental Health Sciences, and the Centers for Disease Control. The Michigan Department of Community Health identified exposed individuals in 1976–77 and obtained baseline health information and blood serum samples. Information on growth and pubertal development was collected by Emory University investigators from 308 daughters born to PBB-exposed women through telephone interviews and medical records.

Puberty began earlier in girls exposed to PBBs in utero and through breastfeeding.<sup>23</sup> Breastfed girls exposed to high levels of PBB in utero ( $\geq 7$  ppb) had an earlier age at menarche (mean age = 11.6 years) than breastfed girls exposed to medium levels of PBB in utero (mean age = 12.2 years) and breastfed girls exposed to low levels of PBB in utero (mean age = 12.6 years). No association was observed between prenatal PBB exposure and daughters' height or weight adjusted for height.<sup>24</sup>

Normal pubertal development involves thyroid hormones, and animal studies indicate that exposures to PBB during pregnancy and lactation causes decreased thyroxine levels in young

offspring. Because five of the daughters in the study reported thyroid problems, PBB-induced early onset of puberty may involve the thyroid gland.

The results reveal that early developmental exposures to EDCs can advance pubertal development. Early puberty in girls is associated with higher rates of behavioral problems and earlier initiation of alcohol use, sexual behavior, and pregnancy.

### ***Growth and Development in Boys Exposed to Dioxin in Chapaevsk, Russia***

Dioxins are potent developmental and reproductive toxins in animal systems, but data on the effects of early exposures to these chemicals in human populations are lacking. Human exposures to dioxins are generally low in North America, but industrial activity has resulted in areas of significant contamination elsewhere in the world. In Chapaevsk, Russia, documented high exposure to dioxins exists in an area surrounding a chemical plant. STAR grant research in progress at the Harvard University School of Public Health is characterizing the association between dioxin exposure, physical growth, and timing of pubertal development in boys. The project is monitoring a cohort of 500 Chapaevsk boys aged 8–9 years exposed to dioxins from the plant.<sup>25, 26</sup> The researchers will continue to characterize adverse developmental and reproductive effects associated with the exposures of boys until the age of 18.

In the studies published to date, Chapaevsk boys were thinner than boys from the United States and other Russian boys in general, had a later onset of puberty, and matured later than boys from other countries. Older boys who ate local meats and fish had higher levels of dioxins in the blood serum. These results provide insights into dioxin exposure patterns, which suggested that the majority of dioxin exposure is dietary, and highlight the importance of investigating the potential health consequences of exposures to dioxin and dioxin-like compounds in children and adolescents.

### ***Blood Lead Concentration and Timing of Puberty in Girls***

Metals may act as endocrine disruptors through their interactions with hormone receptors. Divalent metals, including lead, copper, and mercury, have been shown to bind to estrogen receptors with a high affinity, promoting receptor activation and inhibiting binding with the natural estrogen. With respect to lead, animal studies indicate that it alters hormone concentrations and delays puberty in rats. Children with increased blood lead concentrations have decreased height, weight, or both. ORD scientists hypothesized that exposure to lead may change the timing of puberty through effects on development of the endocrine system and/or on growth. They investigated relationships between blood lead concentration and pubertal development among girls aged 8 to 18 years.<sup>27</sup> The investigators analyzed blood lead concentration data and timing of puberty stages (i.e., age at menarche, breast development, and growth of pubic hair) collected from 2,186 girls enrolled in the third National Health and Nutrition Examination Survey conducted from 1988 to 1994. The study evaluated three ethnic groups separately, because previous studies indicate that the timing of pubertal development differs between ethnic groups. After adjusting for body size, age and potential confounders, the investigators found that blood lead concentrations of 3 micrograms per deciliter of blood ( $\mu\text{g}/\text{dL}$ ) were associated with significant delays in breast and pubic hair growth in African-American and Mexican-American girls compared to girls of the same ethnicity with blood lead levels of 1  $\mu\text{g}/\text{dL}$ . The delays in the African-American group were most significant; delays in 4 breast developmental stages ranged from 2.2 to 6.0 months. There were smaller stage-specific delays in Mexican-Americans, and nonsignificant delays in whites. The delay in age at menarche was also

significant for African-American girls (3.6 months). No significant delays in age at menarche were observed for Mexican-Americans and whites.

EPA considered this study in its revised Air Quality Criteria for Lead<sup>28</sup> in which the health and environmental effects of ambient air lead exposures were critically assessed. Even though the observed associations between lead exposure and female growth and reproductive outcomes are fairly small, sensitive subpopulations may be susceptible to reproductive effects of lead.

## **Effects on Fertility**

Formation of gametes and other reproductive events, such as ovulation, are controlled by hormones. The ability of sperm to fertilize an egg is controlled by many molecular and cellular functions that could be sensitive to disruption by hormonally-active chemicals. Several of these functions are essential to conception, such as the sperms' mobility to reach the egg or its ability to penetrate and fuse with the egg's membrane. Others are critical for normal development of the embryo, including the integrity of the sperm's nucleus and DNA. While it has been speculated that exposure to EDCs contributes to human infertility and/or declines in semen quality and sperm count, no linkage has been established to date between exposure to a specific environmental EDC and these adverse health outcomes in humans. EPA's research on the extent to which EDCs exposures are associated with effects on fertility among EDC-exposed adults or offspring exposed in utero through laboratory animal testing and epidemiologic studies. The research also developed animal models that enhance the ability to assess the potential of EDCs to produce this disease in human populations.

### ***Endocrine Disruptors Observed to Affect Male Rat Fertility Across Multiple Generations***

In the early 1990s, the relationship between exposure to endocrine disruptors and human male fertility was uncertain. Controversial data suggested that average sperm count was decreasing in human populations. Researchers at Washington State University examined the effects of vinclozolin and methoxychlor on testicular cell growth and differentiation during early development and whether these effects reduce male fertility and sperm production later in life or in subsequent generations. Although the doses tested were higher than those expected in the environment, the findings suggest a re-programming of the male germ line in response to endocrine disruption that persisted through generations even though subsequent generations were not exposed. The studies suggest a new mechanism of endocrine disruption during early development that could lead to fertility problems in adult offspring and adult offspring of subsequent generations.

Only the first generation of male rats were exposed in utero to the test chemicals via dosing their mothers intraperitoneally with the test chemicals during gestation. Indicators of fertility of the male offspring the first and three subsequent un-treated generations were studied.<sup>29</sup> The vinclozolin-treated groups (100 mg/kg/day) exhibited adverse effects on fertility in the first generation of males and subsequent generations. The transgenerational effects observed included two-fold increase in spermatogenic cell apoptosis (programmed cell death) through four generations. Sperm numbers were decreased 20%, and sperm motility was reduced about 25 to 35%. Methoxychlor-treated animals (200 mg/kg/day) exhibited similar decreased spermatogenic capacity and sperm viability in the first and second generations. The researchers found altered

DNA methylation patterns in male offspring of vinclozolin and methoxychlor-treated rats compared to controls, suggesting a mechanism for the transgenerational effects.

### ***Endocrine Disruptor Effects on Fertilization and Pregnancy Outcomes in Female Rats***

In rats, humans, and other spontaneously ovulating mammals, the final stages of ovarian follicular and egg maturation and ovulation is stimulated by a sharp mid-cycle rise in luteinizing hormone. The timing of the events is important for reproduction, but the surge can be inhibited by certain chemicals if they are administered during a critical period immediately prior to the surge. In the early 1990s, ORD scientists studied chemicals that inhibited ovulation, and found that thiram, a dithiocarbamate fungicide used in seed treatment, blocked ovulation in rats when a single dose as low as 12 mg/kg was administered during the critical period.<sup>30</sup> They determined that although ovulation was delayed, the number of eggs did not differ from controls but the treated mothers produced smaller litters.<sup>31</sup> The reduced litter size was not attributable to a direct effect of the compound on the oocytes or cells that develop into eggs. The study revealed a new mode of action for EDC effects on fertility.

To examine whether the decreases in litter size were a consequence of aging of the oocyte in the follicle of the ovary, oocytes, fertilized eggs (zygotes), and two-cell embryos were collected from thiram-exposed female rats and examined for fertilization success and abnormalities.<sup>32</sup> Changes included a significant decrease in percentage of fertilized eggs. No apparent abnormalities were observed in the unfertilized mature eggs released following the delay in ovulation, but they were not as successful as controls when the sperm interacted with the egg. A significant increase was observed in the number of eggs fertilized by two or more sperm, a condition called polyspermy, which leads to death of the embryo. The treated-animals' fertilized eggs exhibited an altered pattern of the polysaccharide bodies embedded their outer cell layer, which suggests a disruption of the function that prevents other sperm from continuing to fertilize an egg after it has been fertilized already.

The results demonstrate that a pesticide-induced, 24 hour delay in ovulation altered the fertilization success of released rat oocytes and increased the likelihood of polyspermy, thereby affecting pregnancy outcome. These findings are relevant to other EDCs that delay ovulation. In addition, although rat and human ovarian cycle lengths differ, considerable homology exists between these species in the central nervous system mechanisms controlling ovulation and fertilization. There is some indirect evidence from in vitro fertilization studies that increased intra-follicular aging of human eggs may be associated with an increased rate of polyspermy. Therefore, these findings in rat models may be applicable to humans.

### **Thyroid Hormone Disruption and Neurological Effects**

Maintenance of normal thyroid hormones levels is essential for development of the nervous system of the fetus. It has been observed that when thyroid hormones are severely reduced in pregnant women, in a condition called hypothyroidism, their children are born with mental deficits. Significantly less understood, however, are potential effects on early development from low levels of thyroid hormone disruption that might arise from exposure to environmental contaminants. An understanding of and methods for assessing the effects of mixtures of thyroid disrupting chemicals are also needed by EPA to protect human health. EDRP has made significant progress on both of these research needs.

## **Reducing Uncertainties of Low-level Thyroid Hormone Disruption**

Low-dose extrapolation from rodent studies to humans is a major uncertainty in risk assessment for thyroid disrupting chemicals. An ORD research project aimed to develop dose-response data for a number of thyroid disrupting chemicals to determine the effects of marginal or mild reductions in the levels thyroid hormones. This research will lead to improved risk assessment of thyroid disrupting chemicals by defining the degree of thyroid hormone insufficiency that is without adverse outcome and decreasing the uncertainty in interpretation of changes thyroid hormone. Results will be used to generate quantitative models relating hormone disruption to neurological outcome at different life stages in rats and humans.

ORD scientists demonstrated a relationship between developmental disruption of thyroid hormones and adverse structural and functional effects on the auditory system.<sup>33</sup> The magnitude of hearing loss is correlated with the degree of developmental hormone disruption and the findings revealed an approximately 50% reduction in circulating levels of thyroid hormone is needed to produce hearing loss in rodents.<sup>34</sup> Although hearing loss is reported in humans with disruptions of the thyroid axis, impairments in other functional domains are evident in children in the absence of auditory deficits. Therefore, ORD's research focused on neurodevelopmental effects that may occur in response to more subtle disruption of thyroid hormones (i.e., cognitive function) and may be more relevant to that induced by environmental contaminants in humans.

ORD studied functional consequences of mild thyroid hormone disruption on brain development, the molecular biology underlying thyroid-responsive brain development and function, and identified biomarkers of adverse effects. Pregnant and lactating rats were treated from gestation day 6 through postnatal day 30 with propylthiouracil, a chemical that interferes with thyroid hormone synthesis.<sup>35,36</sup> The study showed that thyroid hormone insufficiency disturbs synaptic function in a brain region that is critically involved in learning and memory. Graded levels of thyroid hormone disruption produce dose-dependent reductions in synaptic function in offspring of maternal rats with low thyroid levels. Electrophysiological impairments at the synaptic level were associated with deficits in learning after the hormone levels had recovered. The observations showed that transient exposure to thyroid hormone disruption during a critical time period of development can lead to adverse effects on learning.

ORD scientists identified a malformation in the developing rat brain that persists to adulthood and derives from a transient reduction in thyroid hormone late in gestation.<sup>37</sup> The structural abnormality was found within the white matter of the corpus callosum of both hemispheres. The structural abnormality was seen at modest levels of maternal thyroid hormone insufficiency—approximately 45% reductions in T<sub>4</sub> with no change in T<sub>3</sub>. The malformation consisted of neurons formed between gestational days 17 to 19 and increased in size with decreases in thyroid hormone levels. It persisted in adult offspring despite a return to normal hormonal status. The investigators hypothesized that this malformation occurs from errors in cell migration that occur as a result of altered thyroid hormone signaling. The long-term consequence of this malformation on brain function remains to be determined, but its occurrence highlights the critical role that thyroid hormone plays in brain development during the prenatal period. In humans, such brain

### **Thyroid Hormones**

The thyroid hormones thyroxin (T<sub>4</sub>) and triiodothyronine (T<sub>3</sub>) are produced in the thyroid. Major functions of these hormones include stimulating oxidative metabolism and regulating growth and development.

anomalies are often associated with a proclivity to developmental disorders including childhood epilepsy.

### ***Assessing Effects of Mixtures of Thyroid Disruptors***

Humans are exposed to complex mixtures of chemicals both natural and synthetic throughout life. It has been a long-standing challenge for human health risk assessment to determine the potential effects from exposure to mixtures of chemicals. EDCs, with their complex modes of action, are particularly difficult. ORD conducted studies to characterize interactive effects of multiple thyroid disrupting chemicals. This research supports methods for predicting the effects of thyroid-disrupting chemical mixtures without directly testing the mixture.

ORD scientists tested whether additivity theory or a toxicity equivalency factor approach predicts the effects of a mixture of thyroid hormone disruptors that increase the breaking down of thyroid hormone through interactions with receptors in the liver.<sup>38</sup> They measured thyroid hormone levels in young female rats exposed to individual chemicals and to a mixture of 18 polyhalogenated aromatic hydrocarbons. The chemicals tested included polychlorinated dioxins, furans, and PCBs. The results indicated that dose additivity predicts the effects of a mixture of 18 chemicals at low doses. At higher doses significantly above typical human “background” exposures, there was a greater than additive effect. The data suggest that the dose addition could be used for low-dose cumulative risk assessments for thyroid disruptors, but at higher doses, assessments will need to consider possible interactions between the individual agents being evaluated. These results were presented to EPA’s Office of Water and the Office of Pesticide Programs to support their evaluation of cumulative risks associated with exposure to thyroid hormone disruptors.

### **Impacts and Outcomes of Research**

EPA’s Endocrine Disruptor Research program contributed significantly to the state-of-the-science for understanding outcomes of endocrine disruption during early development and puberty. Impacts and outcomes include the following:

- data that supported updates to EPA human health dose-response assessments for two pesticides (vinclozolin and atrazine), as well as lead, dibutyl phthalate, and brominated diphenyl ethers;
- improved understanding of mechanisms of adverse reproductive effects of EDCs following early developmental exposure;
- increased knowledge of susceptibilities to EDC exposures during puberty and adverse effects on growth and sexual maturation;
- greater understanding of the impacts of maternal thyroid disruption and development of neurological effects in children, which will improve risk assessments for thyroid toxicants;
- novel models that reduce mammalian animal use and associated costs and enable detailed study of EDC mechanisms of early developmental or other adverse effects; and
- methods for assessing cumulative hazards of mixtures of EDCs, including reproductive effects of dioxin and PCBs and thyroid disruption from mixtures of thyroid toxicants.

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## CHAPTER 4. EXPOSURE AND EFFECTS IN WILDLIFE

Wildlife species may be especially vulnerable to the effects of endocrine disruptors due to their higher potential for contact with endocrine disrupting chemicals (EDCs) in soils, sediments, surface waters, or through their diets. The results of these exposures are often observable in wildlife populations because of either their particular biological sensitivities to EDCs, differences in endocrine systems, or their higher exposures. However, differences in the endocrine systems of wildlife species from the systems of laboratory animals make it difficult to extrapolate study results from the laboratory to the field and accurately characterize the toxic effects from mixtures of EDCs.

To address these uncertainties the focus of Endocrine Disruptor Research Program is to conduct and fund research to understand:

- Sources of EDCs in the environment.
- Effects of exposure to EDCs on vertebrate and invertebrate animals.
- Differences in species sensitivity to EDCs.
- Effects on wildlife populations.

“The EDC researchers are a group of highly esteemed scientists who are at the forefront of EDC research in screening and testing methodologies for mammalian and ecological tests, source identification, effects on wildlife, and ecological health.”

— EPA Board of Scientific Counselors, Subcommittee on EDCs, *Endocrine Disrupting Chemicals Research Program Review*, April 2005

### Identifying Sources of EDCs in the Environment

Research performed under the Endocrine Disruptor Research Program has made significant progress in identifying sources of EDCs, characterizing their environmental concentrations, and assessing their potential to adversely affect wildlife. The program has characterized sources of EDCs including surface water discharges from wastewater treatment plants, concentrated animal feeding operations, and pulp and paper mills.

#### **Wastewater Treatment Plants**

Municipal sewage can contain estrogenic compounds, including natural human estrogens, pharmaceutical estrogens, and household and industrial chemicals. Wastewater treatment plants treat wastewater so that it can be safely returned into the environment according to state water quality standards. Evidence suggests estrogenic compounds are being discharged to surface water bodies and affect fish that live downstream from the plants. ORD researchers have observed male carp (*Cyprinus carpio*) and male walleye (*Stizosteidon vitreum*) collected downstream from treatment plants with significantly elevated serum egg protein (vitellogenin) concentrations and significantly decreased serum testosterone concentrations compared to fish collected from an unaffected river in a national park.<sup>1,2</sup> While many wastewater treatment plants discharge estrogenic compounds, the problem is not universal—the University of Alabama analyzed male mosquitofish (*Gambusia affinis*) downstream from a wastewater treatment plant, and the fish did not show any biological changes indicating exposure to estrogens.<sup>3</sup> ORD has undertaken a significant research effort to address this issue and determine why certain types of wastewater treatment plants or their operations are more effective in removing estrogenic compounds.

To detect estrogenic activity, ORD scientists developed and validated an assay, the fathead minnow (*Pimephales promelas*) vitellogenin gene expression assay, a near real-time DNA-based molecular indicator method for detecting and characterizing estrogenic activity in surface waters. This method was extended to include marine and coldwater fish species through a collaboration between ORD and Michigan State University to evaluate vitellogenin protein and gene expression in the sheepshead minnow (*Cyprinodon variegates*) and the rainbow trout (*Oncorhynchus mykiss*).<sup>4,5,6</sup>

In an additional collaboration with the University of Cincinnati, ORD scientists developed and refined analytical methods to detect and quantify EDCs in liquid samples along the systems of two pilot-scale municipal wastewater treatment plants.<sup>7</sup> The target analytes included seven sex hormones (estradiol, ethinyl estradiol, estrone, estriol, testosterone, progesterone, and androstenedione) and nonylphenol polyethoxylates and their biodegradation products, nonylphenol and nonylphenol ethoxylates. Improved analytical methods are necessary to support evaluation of complex media containing various EDCs to characterize the lines of evidence and understand which EDCs may cause adverse effects in aquatic organisms.

ORD researchers collaborated with EPA's regional offices and state, tribal, and local authorities to sample wastewater treatment plants, surveying estrogenic activities to compare the relative effectiveness of treatment processes. They used the fathead minnow vitellogenin gene expression method to assay 50 effluents, of which 13 showed increased vitellogenin gene expression in male fathead minnows. ORD scientists also collaborated with the New Mexico Department of Environment to determine potential EDC exposure on the Gallinas River above and below the Las Vegas, New Mexico, water supply. New Mexico and ORD split samples and analyzed 27 potential EDCs, using the new assay to assess estrogenic potential. The analysis of this data will help in the effort to determine why some treatment plants are more successful at removing EDCs than others. The results of the analysis will provide treatment methods and best practices for minimizing the discharge of estrogenic chemicals to aquatic environments downstream of wastewater treatment plants.

### Estrogenic Indicator Technologies

Vitellogenin is a protein that normally precedes the production of egg yolk in female fish. Males have the gene for vitellogenin production, but it is not produced normally. Research has shown that some chemicals can bind to estrogen receptors and activate the production of vitellogenin in male fish.

The measurement of vitellogenin and the expression of vitellogenin genes in male fish are now used widely as indicators of exposure to estrogenic compounds. EPA ORD developed a method to detect these biomarkers of exposure.

ORD conducted five training sessions on using this method for water resource managers in EPA regions, the State of California, and a western tribe.

### Concentrated Animal Feeding Operations

In the United States there are an estimated 238,000 animal feeding operation farms in which animals are raised and held in confinement. These concentrated animal feeding operations (CAFOs) annually produce more than 500 million tons of animal waste.<sup>8</sup> Evidence shows that CAFO waste may introduce into surface and ground waters hormonally active materials that include high-potency natural and synthetic steroids. Manure applications may also affect terrestrial systems. Information is insufficient to assess possible ecological risks of potential

exposures to EDCs from CAFO wastes. The goal of EPA's CAFO research is to characterize the types and levels of hormones in the CAFO waste and assess the ecological effects of the hormones on fish. Results from these studies will be used to quantify the risk CAFOs pose to aquatic life.

To evaluate reproductive effects of these steroid metabolites in fish, ORD scientists conducted experiments with the fathead minnow (*Pimephales promelas*). The researchers exposed fish for 21 days to 17- $\beta$ -trenbolone and found the total number of eggs produced by female fish was significantly reduced at measured concentration levels greater than or equal to 27 nanograms per liter of water (ng/L) compared to controls.<sup>9</sup> Females exposed to these levels also exhibited physical characteristics normally present only in mature males. A 21-day fathead minnow study of 17- $\alpha$ -trenbolone showed similar effects in females at comparable exposure concentrations even though this form of metabolite shows a lower binding affinity for mammalian androgen receptors compared to the  $\beta$  form.<sup>10</sup> The similarity in potency may be attributable to the observation that the fish convert a substantial amount of the 17- $\alpha$ -trenbolone to 17- $\beta$ -trenbolone; tissue concentrations of the  $\beta$  form were similar to or greater than the concentrations of the  $\alpha$  form. These results show that characterizing levels of both trenbolone metabolites is important for assessing the potential ecological risk of androgens associated with beef CAFO effluents.

ORD researchers sampled water runoff discharges from a beef CAFO and in laboratory tests found significant androgenic activity; substances in the effluent bound with and activated androgen receptors in a cell-based assay.<sup>11</sup> In the same study, researchers analyzed the runoff samples for two metabolites of a synthetic anabolic steroid, trenbolone acetate, which is administered to cattle as a growth promoter to enhance beef production. One of the two metabolites, 17- $\alpha$ -trenbolone, was detected in two-thirds of the runoff samples: five of the samples exceeded 25 ng/L, and three exceeded 75 ng/L, which are above the levels at which effects were observed in the laboratory. The other metabolite, 17- $\beta$ -trenbolone, was detected in two of the nine samples at concentrations of 10 and 20 ng/L. These metabolites were also detected in seven of the samples of the river receiving the runoff, although at much lower levels. The study provided a well-documented example of the occurrence of trenbolone metabolites in beef CAFO runoff and in a U.S. river receiving the runoff.

### **Pulp and Paper Mills**

Pulp and paper mill effluent has been suspected of contributing to the masculinization of fish, in which female fish grow a gonopodium, a modified anal fin typically found only in male fish. In the Fenholloway River in Florida, 80% of female mosquitofish (*Gambusia holbrooki*) were partially masculinized and 10% completely so.<sup>12</sup> University of Alabama at Birmingham investigators evaluated potential causes of the masculinization effects in mosquitofish. The investigators identified the presence of effluent chemicals that bind with androgen receptors and mimic the responses of androgens that naturally occur in the body. The research results showed that androstenedione, a precursor to active androgens that originates from pine trees used to produce paper, was found at a mean concentration of 0.04  $\mu\text{g/L}$  in the Fenholloway River water column.<sup>13</sup> The sediment mean concentration was 0.7  $\mu\text{g/L}$ . Progesterone, a precursor of androstenedione, was measured in river water at a mean concentration of 2.06  $\mu\text{g/L}$  and in the sediments at 48.8  $\mu\text{g/L}$ . These steroid concentrations dramatically exceed levels observed at Spring Creek, a river tributary that does not have masculinized mosquitofish or receive paper mill effluent. Androstenedione was not detected at any level in Spring Creek, while relatively

low levels of progesterone were detected in the sediments at a mean concentration of 0.09 µg/L, and no levels were detected in the water. The results support the hypothesis that initial pulp-derived steroid compounds that are released by the pulp and paper mill processes accumulate in the sediments where they are transformed by microbes into androstenedione and other androgens that may be contributing to the occurrence of masculinized mosquitofish.

In light of those findings, ORD scientists used laboratory tests to evaluate whether androstenedione contributes to observed androgenic activity of the pulp mill effluent. The researchers separated the pulp mill effluent into separate parts or fractions for analysis and assessed them for human androgen receptor activity in a cell-based system.<sup>14</sup> After analyzing the androgen-active fractions, the researchers did not detect androstenedione in the fractions. Further work is needed to identify additional androgenic substances in pulp and paper mill effluents to characterize the potential for ecological risks.

## **Effects of Exposure to EDCs in Vertebrate Wildlife**

Many studies have been conducted on rats and mice which are mammalian vertebrates based on their relevance for human health, but other ecologically- or economically-important vertebrate wildlife may be sensitive to endocrine disruption. EPA's Endocrine Disruptor Research Program projects evaluated effects of exposure to EDCs for birds, frogs, and fish.

### ***Bird Development and Behavior Are Sensitive to EDCs***

Birds have reproductive strategies and metabolic characteristics quite different from those of mammals and other vertebrates. Exposure to estrogenic chemicals during their development may permanently alter the structure and subsequent functioning of the brain and reproductive systems. Researchers set out to improve understanding of the effects of EDCs on different bird species, including zebra finches, Japanese quail, and chickens.

Investigators at the University of California at Davis researched estrogenic disruption of reproductive system differentiation and development of areas of the brain that differ between the sexes. To investigate the effects of estrogen exposure on the development of altricial birds (i.e., the birds are born helpless after hatching), the researchers administered oral doses of estradiol benzoate—an ester of the naturally occurring estrogen in mammals—to zebra finch nestlings (*Taeniopygia guttata*). The study found that the ingestion of estradiol benzoate can result in adverse effects on development and reproductive success. When female zebra finch nestlings ingested estradiol benzoate at dose levels of 1 to 1,000 nanomoles per gram of body mass (nmol/g) per day, brain masculinization occurred—doses of estradiol benzoate at 10 nmol/g body mass and higher increased the size of regions of brain cells that regulate song control, a typical male behavior.<sup>15</sup> They tested the industrial surfactant nonylphenol and the pesticide methoxychlor at doses of 1,000 nmol/g body mass per day and the pesticide dicofol at 100 nmol/day, but found that these exposures did not influence the formation of song control regions in the brain. In another study by this research team, estradiol benzoate exposure impeded reproductive success of zebra finches. When researchers exposed mating pairs of zebra finches to 10 nmol/g body mass per day of estradiol benzoate for 6 days, egg production, egg survival, and hatching success were all reduced.<sup>16</sup> The researchers exposed zebra finches to octylphenol at dose levels of 10 and 100 nmol/g, but they did not detect any adverse effects on reproductive performance.

In a similar study, University of Maryland scientists investigated the effects of estradiol benzoate and vinclozolin, an anti-androgenic fungicide, on Japanese quail (*Coturnix japonica*). The investigators injected Japanese quail eggs with estradiol benzoate and evaluated reproductive capability and success (e.g., fertility, hatching success, behavioral responses, and plasma steroids).<sup>17</sup> They observed that it affected endocrine and behavioral responses in males and affected egg production in females, results that were similar to the zebra finch findings. In another study, quail embryos were exposed to vinclozolin by injecting 25 to 100 parts per million (ppm) of the chemical into eggs. Males showed significantly lower levels of gonadotropin-releasing hormone, a hormone released by the hypothalamus that controls sperm production. The reproductive behavior of the quail was often delayed compared to controls, with fewer attempted copulatory mounts and contacts with other birds' intestinal, urinary, and genital tract openings.<sup>18</sup>

Investigators at the University of California at Davis evaluated sex-related differences in biochemical and toxic effects of exposure to dioxin using chickens as a model. Immature male and female chickens were exposed to either 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), estrogen, or both TCDD and estrogen. Female chickens were more sensitive than males to TCDD-induced wasting syndrome, characterized by prolonged weight loss, when exposed to doses of 10 and 100 microgram ( $\mu\text{g}$ ) TCDD/kilogram (kg) body mass.<sup>19</sup> Fat tissue mass, liver mass, and body weight increased when immature male chicks were exposed to TCDD, although these increases were no higher than increases for chicks exposed to TCDD and estrogen.<sup>20</sup> Because estrogen did not increase the toxicity of TCDD in immature males, the higher sensitivity of female chickens to TCDD may be related to tissue-specific factors not present in the estrogenized male chickens within the 10 days of exposure.

These studies of zebra finches, Japanese quail, and chickens identified exposure indicators, such as brain and reproductive function, which can be measured and evaluated to detect endocrine disruption in birds. The results also showed that embryos were most sensitive to developmental effects. Exposure to an estrogen derivative resulted in adverse effects on reproduction and masculinization of females brain structures. Exposure to the TCDD and vinclozolin induced adverse effects in birds, while studies of other suspected EDCs, including octylphenol, methoxychlor, and dicofol did not detect adverse effects at the dose levels tested.

### ***Retinoid Disruption as Potential Cause of Frog Malformations in the Wild***

Beginning in the 1990s, investigators observed an increased frequency of hind-limb malformations—extra, missing, truncated, or malformed rear limbs—in wild frogs in the upper Midwest of the United States and Canada. These amphibian species also experienced declining numbers worldwide. Although many hypotheses offer explanations of the cause of these deformities and population effects, evidence of a cause and effect relationship is inconclusive. Due to the strong similarities between effects observed in the field and effects of retinoid signaling disruption observed in the laboratory, one theory proposes that frogs are exposed to a waterborne contaminant that disrupts sensitive cell-signaling biochemical reactions controlled by retinoids. Retinoids are metabolites of vitamin A that regulate processes critical to embryonic development such as cellular differentiation and tissue proliferation. Retinoids bind with retinoic acid receptors and steroid receptors in the thyroid to regulate expression of genes for growth and development. University of California investigators analyzed chemicals to begin identifying whether retinoid-active compounds are present in the environment where deformed frogs are found routinely.<sup>21</sup> They found unknown retinoid-active substances in water samples taken from

two locations where frog malformations have been observed, and their investigation is continuing in other locations in the United States.

To test the theory of retinoid disruption, ORD scientists examined developmental effects in five species of frogs following their exposures to a retinoid, all-trans retinoic acid, an acidic form of vitamin A.<sup>22</sup> The scientists selected this retinoid model for study because it has a high affinity for retinoic acid receptors, and other retinoids frequently are compared with it. The experiments involved exposing the frogs at certain times of their development (i.e., during the midblastula embryonic stage) for three of the species and during tadpole stages for four of the species. Embryos exposed to less than lethal concentrations (2 µg/L) did not result in body structure abnormalities including hind limbs in the four native North American species tested. The tadpole exposures resulted in abnormal effects on the front part of the body but not the rear limbs. This result indicates that exposure to an environmental retinoid is not responsible for hind limb malformations observed in the field, but that all-trans retinoic acid is more toxic in the embryonic stage than the tadpole stage. Under continuous exposure to retinoid, embryos would not survive to develop to the tadpole life stage, during which limb deformities are triggered. ORD scientists confirmed these findings in a follow up study that showed continuous, long-term exposures of frog embryos to all-trans retinoic acid resulted in mortality, not limb malformations.<sup>23</sup>

ORD studied effects of retinoic acid on the wood frog (*Rana sylvatica*) which is native to North America. Other native species tested included the green frog (*R. clamitans*), the northern leopard frog (*R. pipiens*), and the mink frog (*R. septentrionalis*).

Researchers at the University of California at Irvine exposed African clawed frogs (*Xenopus laevis*) to another retinoid model, TTNPB\*, a synthetic chemical known to activate the retinoic acid receptor in amphibians and other vertebrates.<sup>24</sup> The researchers' results were consistent with ORD's findings in that embryos exposed to TTNPB experienced death but no malformations, while tadpoles exposed during certain developmental stages developed duplicated limb buds and other limb malformations. These findings support the conclusion that it is unlikely that retinoid mimics produce the range of limb abnormalities found in wild frogs.

ORD scientists evaluated another chemical's potential to cause malformations in frogs. Researchers tested the pesticide methoprene and its degradation products to determine whether exposures to these substances during early life stages could lead to limb malformations in African clawed frogs.<sup>25</sup> Methoprene is used in mosquito control; it mimics insect growth hormone to prevent maturity of mosquito larvae. Frog embryos exposed to methoprene for 4 days showed no evidence of developmental toxicity at exposure levels up to 2 mg/L concentration; however, three methoprene degradation products were toxic at the following levels: 1.25 mg/L for methoprene acid, 2.5 mg/L for 7-methoxycitronellal, and 5 mg/L for methoprene epoxide. Methoprene acid caused abnormal head tissue development and swelling; methoprene epoxide and 7-methoxycitronellal resulted in delayed development and swelling. However, other studies of methoprene application rates and persistence in water indicate that it is unlikely that methoprene degradation products will accumulate to these toxic levels, making methoprene an unlikely contributor to frog malformations observed in the environment.

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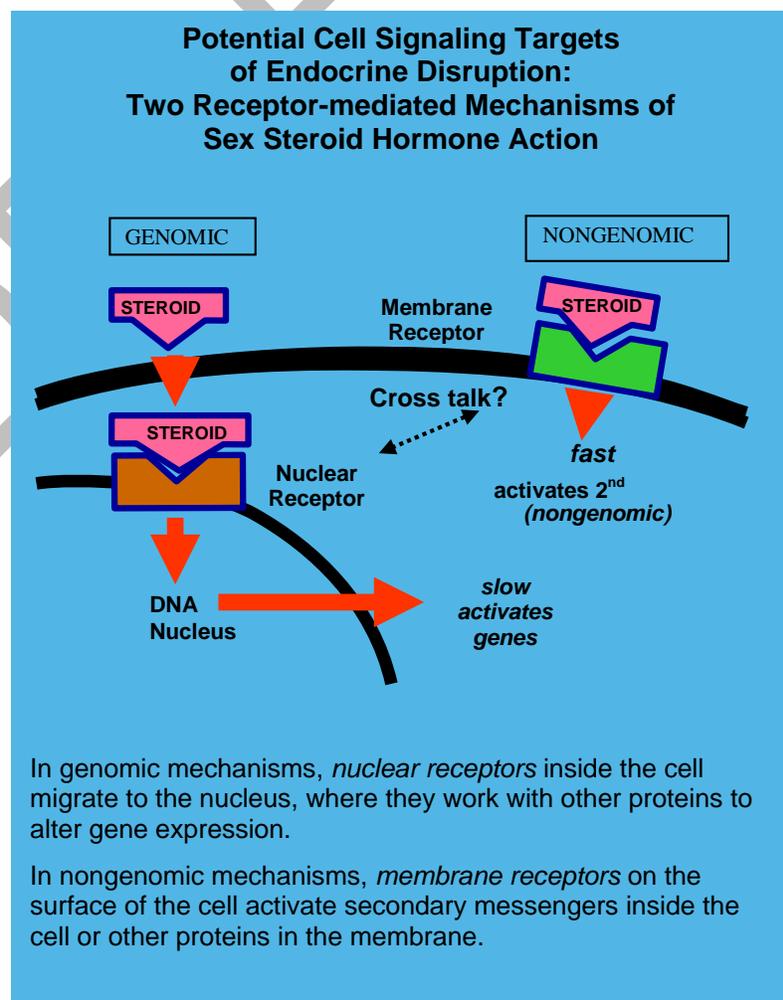
\* The chemical name for TTNPB is 4-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid.

Results of frog studies from the Endocrine Disruptors Research Program demonstrate a period of sensitivity for limb development during the tadpole life stage that is remarkably narrow. Laboratory studies found that model retinoids cause malformation at low doses; however the life-stage differences in sensitivity suggest that exposure timing in the wild would need to be precisely intermittent to produce such effects. The results also indicate that the pesticide methoprene's degradation products can produce malformations, but at levels higher than those that achieved in the environment. It is unlikely that these substances—retinoids and methoprene—are the primary cause of frog malformations in the wild.

### **Multiple Fish Hormone Receptors and Functions Are Potential Targets of Endocrine Disruption**

Research has shown that estrogenic substances in wastewater discharges cause feminization and androgenic substances in paper mill and CAFO effluents cause masculinization in fish. EPA's Endocrine Disruptor Research Program researched the mechanisms of these chemical effects and their implications for wild fish populations. The research seeks to improve knowledge of reproductive biology and endocrinology of different fish species, evaluate the biological changes that lead to the development of adverse effects, and determine the levels at which adverse effects occur. EPA and states need this information to identify safe EDC exposure levels for aquatic life and to develop ecological risk assessments that inform regulatory decisions regarding control of wastes containing EDCs.

A study by scientists at the University of Texas identified sex steroid hormone receptors and compared the reproductive and endocrine toxicity of a representative estrogenic EDC (o,p'-DDT) and an anti-androgenic EDC (p,p'-DDE) in Atlantic croaker (*Micropogonias undulatus*) to illuminate how EDCs disrupt hormone signaling pathways. The Atlantic croaker makes a good study model based on its well characterized physiology and its importance as a sport fish and prey species in estuarine and marine waters. The researchers identified three genetically distinct estrogen receptors and two genetically distinct androgen receptors in female and male croakers, each with a different binding affinity for estrogens and anti-androgens.<sup>26,27,28</sup> Differences



in receptor binding affinity were affected by exposure to EDCs. Differences in receptor function were based on their location, either inside the nucleus or along the membrane, in the cells of reproductive tissue. The tested chemicals o,p'-DDT and p,p'-DDE, along with other EDCs, showed different binding affinities within the range of receptors, reacting with some receptors but not with others. The study suggests that these DDT-derivatives bind with an estrogen membrane receptor, which alters androgen production, thus leading to male reproductive effects (i.e., a nongenomic mechanism). These substances also bind with nuclear estrogen receptors to activate gene expression; however, in Atlantic croaker this genomic mechanism appears to be less sensitive

Studies that identify nongenomic mechanisms of endocrine disruption are significant because they indicate that different patterns of biochemical changes, adverse effects, and degrees of impairment in endocrine function can be linked to exposure to estrogen- and androgen-active chemicals. In addition, the results help explain differences in sensitivity between male and female fish and between developmental stages in a species.

### ***Low-Level Estrogenic Chemical Exposure Impairs Minnow Reproduction and Populations in a Whole-lake Study***

Laboratory studies have linked estrogenic chemicals in wastewater to feminization of male fish, but information was needed to link concentrations of estrogenic compounds to quantify the magnitude of the impact on populations of exposed fish. To investigate the effects of long-term exposure to an estrogenic compound on a whole-lake ecosystem, Fisheries and Oceans Canada scientists, in collaboration with ORD investigators and other sponsors, performed a 7-year estrogen-addition study. For 3 years, from 2001 through 2003, one of the experimental lakes (identified as Lake 260) of the Experimental Lakes Area in northwestern Ontario, Canada, was dosed with the synthetic estrogen, 17- $\alpha$ -ethinylestradiol. This compound is a potent estrogen mimic that is the main active ingredient in birth control pills. Additions of this chemical to the lake were performed to achieve constant and environmentally relevant concentrations in the water level of approximately 5 to 6 nanograms per liter (ng/L) in the lake (i.e., levels similar to those found downstream of wastewater treatment plants in North America).

ORD and collaborating researchers monitored several biochemical parameters (e.g., sex hormone levels, vitellogenin levels) and sex organ and egg development and other indicators of reproductive fitness in fish. Species tested were the pearl dace (*Margariscus margarita*) and fathead minnow (*Pimephales promelas*).<sup>29,30,31</sup> Male and female fathead minnow and pearl dace had elevated whole-body concentrations of vitellogenin within 7 weeks of exposure. Egg development was delayed in both the fathead minnow and pearl dace. Testes development was severely impaired and elements of both male and female gonads were observed in males. Reproductive failure was observed in both of the fish species during the second year of exposure. Kidney lesions in pearl dace were also evident.

The investigators examined the fathead minnow population size and structure in the fall of each of years of the study in both the dosed and reference lakes.<sup>32</sup> While the mean abundance of fathead minnows varied considerably in the reference lake, the abundance of this species of minnows in the treated lake decreased consistently following the ethinylestradiol additions. After the second season of treatment, the fathead minnow population in the treated lake collapsed. Reproductive failures continued in the third season of treatment and for another 2 years after the additions of ethinylestradiol were stopped.

The results from this study suggest that continued exposure of natural or synthetic estrogens and estrogenic compounds to aquatic habitats could decrease the reproductive success and sustainability of fish populations. Results from this whole-lake study may be used to assess the potential risks that hormone mimics pose to fish and fish habitat, and to understand the timing and magnitude of the impacts that estrogen-like compounds have on aquatic organisms. They also provide information that will illuminate relationships between early biochemical changes (biomarkers of exposure) and the development of adverse effects. The study also contributed information that led to the refinement of the vitellogenin gene expression assay developed by ORD.

## **Exposure and Effects on Invertebrates**

While significant research has been conducted to evaluate the potential effects of EDCs in vertebrates, comparatively little research has focused on chemical-induced endocrine disruption in invertebrates although they account for at least 90% of all described animal species. EPA's Endocrine Disruptors Research Program conducted some of the first research on invertebrates to evaluate their sensitivity to EDCs.

### ***Insect Juvenile Hormone Pesticides Evaluated for Effects on Crustaceans***

Insecticide development aims to limit effects on nontarget organisms that live in habitats populated by the target pests. One pesticide class that more selectively targets insect pests is juvenile hormone analogs. These pesticides mimic natural insect juvenile hormone and interrupt insect life cycles by altering embryonic and larval development. There is concern that crustaceans that inhabit mosquito breeding areas are physiologically similar to insects, and they may be sensitive to these pesticides.

North Carolina State University scientists evaluated hormone signaling and effects of insect juvenile hormone in a small aquatic crustacean, the daphnid, or water flea (*Daphnia magna*), reproduction. Daphnids reproduce asexually during periods of abundant resources. When environmental indicators signal the onset of stressful conditions, daphnids produce males and reproduce sexually. The endocrine signals that communicate the switch are unknown. The investigators evaluated whether methyl farnesoate, an insect juvenile development hormone, signals male sex determination in daphnids.<sup>33</sup>

Continuous exposure to this hormone over 21 days caused the daphnids to switch from production of female offspring to the production of male offspring. This raised the possibility that juvenile insect hormone-like substances present in certain insecticides may alter sex ratios of daphnids and other crustaceans with similar reproductive processes. The investigators tested a range of chemicals to determine the effects on daphnid offspring sex ratios.<sup>34</sup> Exposure to pyriproxyfen—an insecticide with a structure similar to a juvenile insect development hormone—increased the percentage of male offspring born among exposed female daphnids. Exposure to the other chemicals tested—the herbicide atrazine, the fungicide fenarimol, an industrial chemical pentachlorophenol, and ethanol used as a solvent for the other chemicals—did not induce this change, which confirmed that the effect was not a generalized stress response. Changes in sex ratios may have implications for the reproductive success of daphnid and other crustacean species with similar reproductive processes.

ORD exposed developing grass shrimp (*Palaemonetes pugio*) to the pesticide fenoxycarb, another juvenile hormone analog.<sup>35</sup> Larval shrimp were found to be the most sensitive life stage.

When exposed through complete larval development to fenoxycarb concentrations of greater than or equal to 4 µg/L, significantly fewer shrimp successfully developed into post larvae. Crabs exposed during only the embryonic phase were not significantly affected. The results indicate that grass shrimp are sensitive to fenoxycarb at concentrations lower than those published for other nontarget organisms such as fish.

ORD studied effects of fenoxycarb on xanthid crabs (*Rhithropanopeus harrisi*).<sup>36</sup> Similar to the shrimp, larval crabs were the most sensitive life stage and exhibited significant mortality when exposed to a water concentration of 48 µg /L fenoxycarb and higher. At the same concentration, the larvae took an average of 3 to 4 days longer than unexposed crabs to develop into mature crabs. If concentration of this pesticide were achieved in estuarine waters, the crabs' population success may be altered.

Effects of crustacean exposure to insect juvenile hormone analog pesticides included mortality, reproductive effects, and delayed development. Long-term exposure in surface waters could adversely affect populations of economically important aquatic species (e.g., crabs and shrimp) and aquatic food webs.

### **How Tributyltin Disrupts Sexual Development of Female Mud Snails**

The organotin compound tributyltin, a primary component of biocides used in antifouling paint on ships, reduces the reproductive success of mud snails (*Ilyanassa obsoleta*) at extremely low exposure levels. The exposed snails experience imposex, a condition in which females develop male sex organs so they have both female and male organs. Researchers attempted to elucidate the mechanisms by which imposex occurs. Two mechanisms have been suggested and investigated.

North Carolina State University researchers investigated whether tributyltin interferes with the metabolism of testosterone and contributes to elevated testosterone levels that are associated with imposex. Female mud snails were exposed for 6 months to levels of tributyltin that have been found in contaminated areas (1.0 and 10 ng/L). These levels induced imposex and elevated testosterone levels in female mud snails.<sup>37</sup> In addition, tributyltin elevated free testosterone levels in snails by decreasing the production or retention of testosterone-fatty acid esters.

Other researchers suggested that decreased testosterone metabolism likely is not the primary mechanism by which imposex is induced, but rather is instrumental in maintaining male sex organs after they are formed. Duke University researchers identified a neurohormone responsible for releasing a brain hormone called penis morphogenic factor (PMF) that signals snails to develop male sexual structures. The researchers dosed snails with four neurohormones and found that the sex behavior regulator APGWamide significantly induced imposex at doses as low as  $4 \times 10^{-16}$  moles per animal.<sup>38</sup> The investigators suggested that tributyltin could act as a potent neurotoxin that induces imposex in mud snails by disrupting the normal release of PMF.

This research provided important information to explain the mechanisms by which endocrine disruptors affect reproductive development in snails. It illustrates the complexity of interactions that might occur between neurohormones and sex steroids in the induction and maintenance of abnormal sexual characteristics in snails.

## **Wildlife Species Differences in Sensitivity to EDCs**

Testing chemicals for endocrine disruption effects on more than a few species would be cost prohibitive. For the most efficient use of research resources, methods are needed to focus testing on sensitive organisms that are important for maintaining ecosystem structure and function, and if we know enough, whose results could be extrapolated to other species. Molecular biology tools may enable screening of a wide range of species without the use of whole animals. Following are some summaries of studies focusing on species differences and how EDCs interact with their endocrine systems.

### ***Mammal and Fish Differences in Atrazine Response***

To explore differences between two vertebrates' responses to the widely-used herbicide atrazine, ORD researchers compared differences uptake and metabolism of atrazine in rats and fish and its effects on the activity of aromatase—an enzyme that transforms androgens to estrogens during a process called steroidogenesis.

In laboratory-cultured cells, atrazine enhances the activity of aromatase. The researchers tested male rats and a marine fish called the cunner (*Tautoglabrus adspersus*). Atrazine was metabolized at different rates in the two species.<sup>39</sup> Rats absorbed atrazine more efficiently but metabolized and eliminated the metabolites faster than the fish. Fish retained notable levels of an atrazine metabolite in the blood more than 5 days post-treatment, while rats had almost no atrazine or metabolites detected in their blood after this time period. ORD studies also showed that rats and fish differed in their response to atrazine at different dose levels and exposure routes. Male rats had increased levels of steroids circulating shortly after exposure to 50 or 200 mg/kg oral doses of atrazine, but no changes in aromatase activity in their brains or gonads.<sup>40</sup> Both male and female fish implanted with 0.1 to 5 mg/kg atrazine had elevated brain aromatase activity but no significant changes in gonad aromatase activity.<sup>41</sup> The atrazine-exposed fish also exhibited significant reduction in egg production, fertility, and viability of offspring compared to controls.

The studies demonstrated that atrazine and likely other EDCs exert different effects on different species, and this presents challenges for extrapolations of EDC effects between species. This research is improving EPA's knowledge of precisely how EDCs interact with endocrine systems of model organisms and the differences between them. Eventually this information may be used to predict the sensitivity of other organisms with similar endocrinology.

### ***Chemical Screening Tools for Species' Hormone Receptor Binding***

To predict whether an endocrine disrupting chemical may have an effect on a particular species, if particular groups of organisms have estrogen or androgen receptors to which the chemical can bind, recognizing that interactions with hormone receptors are only one of the mechanisms of endocrine action. If binding occurs, the strength of the bond, or the affinity of the chemical to the receptor, are potential indicators of the endocrine activity of the compound. ORD researchers are using vertebrate and invertebrate species from various classes to develop estrogen and androgen receptor binding assays that could lead to high throughput assays to prioritize endocrine disruptors for further testing. They have run competitive binding assays with androgen receptors from fathead minnows, rainbow trout, chimpanzees, and humans. Researchers have also conducted comparative binding studies with estrogen receptors from fathead minnow and rainbow trout and acquired genetic coding for the estrogen receptor of the American alligator, Japanese quail, and the salamander.

## **A Cross-Species Mode of Action Information Assessment: A Case Study with Bisphenol A**

Mode of action is the key step in a chemical-induced toxic response that is responsible for the adverse physiological outcome or pathology. ORD conducted a case study application of a cross-species mode of action data analysis approach that holds promise for predicting endocrine disruption mode of action among untested species.<sup>a</sup> This information would improve the extrapolation of toxicity test data between species, support prioritization of testing, and improve the accuracy of ecological hazard assessments for EDCs.

ORD selected bisphenol A (BPA) for the study because data were available on effects for mammalian, non-mammalian, and invertebrate species, and BPA's mode of action—binding and activation of estrogen receptors to transcribe estrogen-responsive genes—has been described for several vertebrate species.

Published developmental and reproductive effects data for BPA were compiled for 16 species representing three animal phyla. The relationship between species relatedness (i.e., how phylogenetically similar the species are) and the BPA mode of action for the species was assessed. The phylogenetic relationships were based on molecular genetics studies. A weight-of-evidence approach considered whole-animal effects data and cell-based mechanistic data to propose mode of actions for different animal species.

Among five classes of vertebrates, the data supported an estrogen agonist mode of action—BPA binds with estrogen receptors to activate gene expression. Thus, for an untested vertebrate species, the estrogen mode of action could be predicted with varying degrees of confidence depending on the vertebrate class. BPA exhibited a higher affinity for estrogen receptor-alpha in nonmammalian species compared to mammals. Some of the vertebrate effects described in the published studies were not explained by this particular mode of action, and the analysis therefore identified BPA mode of action research needs. Data were insufficient to determine or predict the BPA mode of action among mollusks, arthropods, reptiles, and amphibians.

Another benefit of the cross-species mode of action information assessment approach is that it supports the integration of human health and ecological risk assessments. In an integrated risk assessment, risk estimation for humans, biota, and natural resources are combined into a single assessment.<sup>b</sup> Benefits of this integration include improved consistency in assumptions underlying the human health and ecological hazard characterizations and increased potential for the identification of sensitive species for ecological risk assessments.

<sup>a</sup>U.S. EPA. 2005. A Cross-Species Mode of Action Information Assessment: A Case Study of Bisphenol A. National Center for Environmental Assessment, Office of Research and Development. EPA/600/R-05/044F. April.

<sup>b</sup>World Health Organization (WHO). (2001). Integrated Risk Assessment. Report prepared for the WHO/UNEP/ILO International Programme on Chemical Safety. WHO/IPCS/IRA/01/12. Available at: [http://www.who.int/ipcs/publications/new\\_issues/ira/en/index.html](http://www.who.int/ipcs/publications/new_issues/ira/en/index.html).

Similarly, Michigan State University tested the ability of chemicals to bind with human, mouse, chicken, lizard, frog, and rainbow trout estrogen receptors and induce gene expression.<sup>42</sup> Estradiol induced similar responses across the species, but the other chemicals exhibited marked variability in response across the species receptors tested. These cross species comparisons will facilitate the identification of EDCs that may differentially affect specific species. Because binding and gene expression assays do not take into account metabolism, which can change the chemical into a more or less active form, the assays provide information useful for screening and prioritizing subsequent whole-animal studies and characterizing a mechanism of action, but they cannot be used alone in ecological risk assessment.

## **EDC Effects on Wild Populations**

Much of the Endocrine Disruptors Research Program research on the effects of endocrine disruptors has focused on effects in individual organisms; however, ecological risk assessment information is needed on a broader base—what are the effects of EDCs on higher levels of biological organization and whole populations? The Endocrine Disruptors Research Program studied animals in the field to determine if they are being exposed to known EDCs and whether adverse effects are occurring. Researchers developed approaches for extrapolating from effects on an individual species level to effects on a whole population level by evaluating endocrine disruptors at multiple levels of biological organization, and then combining field or laboratory studies with computer models.

### ***Florida Gulf Sharks Evaluated for Organochlorine Exposure and Fertility Effects***

Organochlorine contaminants pose concerns for sharks and other marine predators because these chemicals persist in the environment, biomagnify in the food chain, and have been shown in the laboratory to impair reproduction. Mote Marine Laboratory accomplished one of the first and largest efforts to characterize levels of organochlorine contamination in sharks that reside in polluted coastal areas. They focused on bonnethead shark (*Sphyrna tiburo*) because a previous study indicated that some populations of this shark may be suffering reproductive impairment, and it was hypothesized that organochlorine exposure might be part of the problem. Mote Marine Laboratory found quantifiable levels of polychlorinated biphenyls in the livers of virtually all of the bonnethead sharks they collected from four estuaries on Florida's Gulf coast.<sup>43</sup> DDT and chlordane compounds were the most frequently detected organochlorines. The Florida bonnethead sharks had organochlorine levels significantly lower than those observed for deep-water sharks, but higher than those found in leopard sharks (*Triakis semifasciata*) collected from San Francisco Bay. In a previous study, bonnethead sharks collected from Tampa Bay showed markedly higher rates of infertility compared to Florida Bay sharks, as well as other differences (e.g., Tampa Bay adult female sharks were significantly larger on average compared to those collected from Florida Bay).<sup>44</sup> Organochlorine concentrations in bonnethead shark from Tampa Bay were also higher than those collected from Florida Bay, providing some evidence that these contaminants may be contributing to reduced reproductive success of these animals. The Mote Marine Laboratory researchers are comparing reproductive fitness of sharks collected from the four study sites to clarify more conclusively the relationship between organochlorine exposures and reproductive effects in wild sharks.

### ***Great Lakes Herring Gulls Show Thyroid Response to Polychlorinated Biphenyl Contamination***

Since the 1960s and 1970s the bioaccumulation of polyhalogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs) has been well documented in fish-eating birds that populate the Great Lakes. While PCB exposure is associated with thyroid disruption (hypothyroidism) in mammals, much less is known about the potential effect on birds. Virginia Tech collaborated with the Canadian Wildlife Service to conduct field studies of thyroid function in herring gulls (*Larus argentatus*) that inhabit PCB-contaminated sites on the Great Lakes (Lakes Erie, Ontario, and Huron) and compared them with gulls collected from a noncontaminated marine reference site.<sup>45</sup> The researchers assessed thyroid hormone levels in gulls at three life stages—embryos, pre fledgling chicks, and adults. Gulls at all three stages from sites with PCB contamination had significantly decreased stores of T<sub>4</sub> thyroid hormone in their thyroid glands, and they tended to have enlarged thyroid glands compared to gulls from the reference site; however, levels of T<sub>4</sub>

thyroid hormone circulating in the blood did not differ significantly in the chicks and adults, which indicated that their thyroid status was normal. Embryos were less able to respond to disturbances in thyroid function; slightly less than half of the embryos from PCB sites showed normal T<sub>4</sub> levels in their blood. The findings suggest that gull thyroid function is less sensitive to PCBs compared to data reported for laboratory mammals. They also indicate that most gulls, particularly older ones, can adapt to maintain circulating hormone levels when exposed to PCBs in their environment. The response relies on thyroid hormone stored in the thyroid gland, which diminishes the gulls' capacity to respond to other stressors on the thyroid such as iodine deficiency, adverse weather, or molting.



Wild herring gulls in PCB-contaminated areas in the Great Lakes had lower thyroid hormone reserves in their thyroid glands, but most gull embryos, chicks, and adults were able to maintain normal thyroid levels in their blood.

### **Alligators Show Altered Hormone Levels at Contaminated Organochlorine Pesticides Sites**

In the 1980s scientists detected a significant reduction in the American alligator (*Alligator mississippiensis*) population of Lake Apopka, Florida. The researchers observed decreased numbers of viable alligator eggs, increased hatchling deaths, and a dramatic decline in the numbers of juveniles and smaller phalluses, too. Lake Apopka was contaminated with organochlorine pesticides, and the study findings, along with other studies, drew attention to endocrine disruption as a potential mechanism of adverse reproductive effects in wildlife. ORD and other organizations implemented studies to determine how organochlorine pesticides affect alligator reproductive success.

One area of study was on the effect of organochlorine pesticide exposure on alligator hormones. Scientists observed that alligators from Lake Apopka and other lakes in Florida showed abnormal testosterone levels. ORD, the University of Florida, and North Carolina State University collaborated on a study to evaluate whether organochlorine pesticides disrupt the alligators' ability to biologically transform testosterone.<sup>46</sup> The researchers collected alligators from four lakes known to be contaminated with organochlorine pesticides and a pristine lake and evaluated the activity of alligator liver enzymes that transform testosterone. The activity of two of the enzymes—testosterone hydroxylase and testosterone oxidoreductase—was altered in alligators collected from the contaminated sites. Male and female alligators normally show gender-related differences in the activity of these enzymes; females metabolize testosterone more than males do. The alligators from the contaminated sites did not show significant differences between sexes. The differences could be attributable to contaminants, but other possible explanations include natural variation among populations. It is unclear whether the hormone level differences are large enough to cause reproductive effects, but

Juvenile American alligators from organochlorine pesticide-contaminated lakes in Florida had altered testosterone transformation enzyme activity patterns compared to alligators collected from a pristine lake.

the research contributes information about hormone level variation in wild populations of alligators and supports the identification of potential mechanisms of endocrine disruption in these animals.

### ***Organochlorine Pesticide EDCs Detected in Crocodile Eggs***

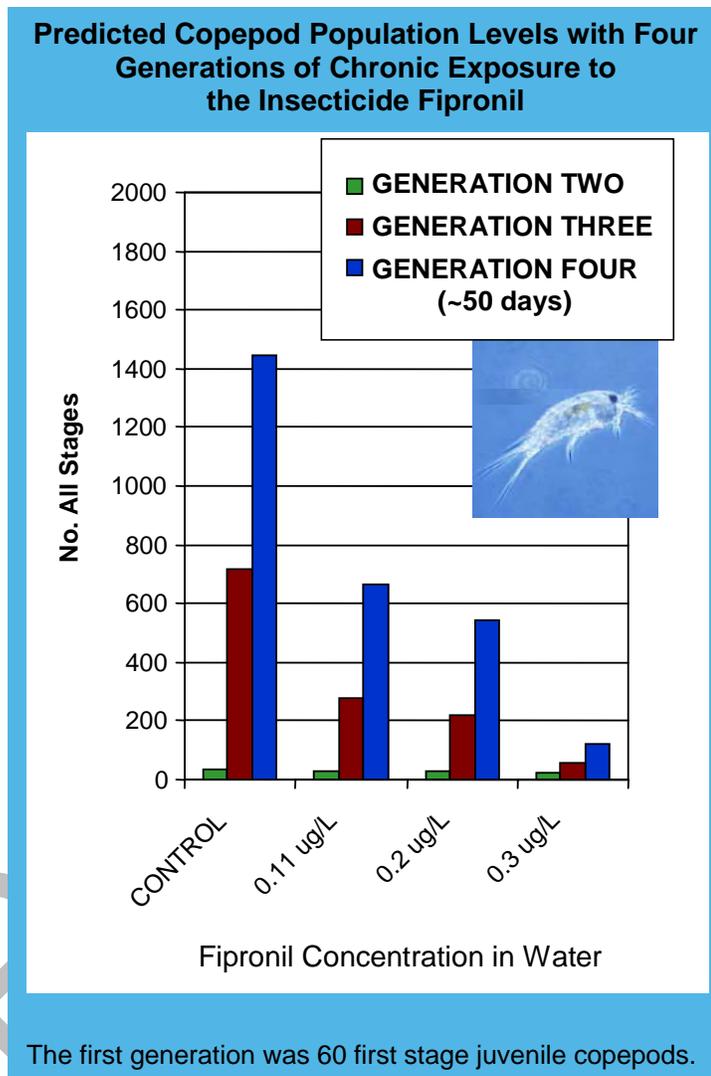
In some developing areas, organochlorine pesticides continue to be applied, and they persist in the environment, bioaccumulating in predators such as alligators and crocodiles, and females transfer the chemicals to their eggs. Texas Tech University researchers conducted field studies in Belize, Central America, to assess EDC concentrations in Morelet's crocodile (*Crocodylus moreletii*) eggs. The researchers found that all of the crocodile eggs tested contained at least one organochlorine contaminant at concentrations ranging from 1 parts per billion (ppb) to 0.7 ppm.<sup>47</sup> The most common contaminants were p,p'-DDE and methoxychlor, which were detected in 100% and 29%, respectively, of the eggs collected. The investigators suggested that the level of organochlorine contamination found in the eggs may pose a subtle long-term risk to endangered crocodiles in Central America, and further research on whether adverse developmental effects are occurring at these levels is needed.

### ***Models from Laboratory Data Predict Population-Level Effects***

Although there is a growing body of ecological toxicity data for EDCs, nearly all of these data describe effects on individual organisms such as reduced survival, growth, reproductive potential, or biological indicators of exposure. Environmental decisionmakers want to know if a chemical affects higher levels of a biological organization because that is a better indicator of the integrity of the ecosystem. If an EDC poses risks to populations and ecosystems, they also want to know how it affects a population's growth rate and how rapidly the population can recover following exposure to an EDC.<sup>48</sup> Effects on population indicators such as population abundance and productivity cannot be predicted directly based on individual-level traits or biomarker data. To compensate for this gap, several research projects developed computer models that estimate population-level impacts from existing toxicity data.

Texas Tech University researchers developed computer modeling techniques to assist in the translation of laboratory experiment results into population-level effects. The research team imported the results of laboratory experiments that estimated PCB effects on fecundity, egg mortality, and predatory avoidance behavior in fish into multiple nested statistical models.<sup>49</sup> The team used one statistical model to estimate the probability of fish survival based on predator avoidance behavior. Then the team used an individual-based model based on the output of the statistical model to estimate larval duration and survivorship at each stage of development. Finally, using the outputs from the individual-based models, the team applied a matrix model to project population growth rates for 100 years based on two hypothetical PCB contamination scenarios.

University of South Carolina scientists developed computer models to predict effects on copepods (*Amphiascus tenuiremis*), small crustaceans that are an important food source for fish. Results of toxicity studies on copepods were included in a predictive matrix computer model which estimates changes in copepod population growth depending on measured changes in reproduction and growth from laboratory tests.<sup>50</sup> For example, the model predicted copepod population sizes assuming that four generations of copepods were exposed to long-term concentrations of the pesticide fipronil in water (0.11 to 0.3 µg/L). The levels tested did not induce mortality but reduced female production of young. The model predicted that the population sizes and population growth rates of the exposed individuals were lower compared to the unexposed groups (see graphic). Information about maintenance of population levels is more meaningful for assessing long-term risks of EDCs to aquatic organisms.



ORD linked biological indicators of endocrine effects to indicators of population-level effects using population models for the fathead minnow (*Pimephales promelas*).<sup>51</sup> A biomarker of exposure to endocrine disruptors (i.e., male plasma phosphorus, which corresponds to vitellogenin production in males) could be linked to measures of survival probabilities or reproductive success, which can then be linked to population modeling predictions. The approach described was applied to projecting the effects of EDCs on the population parameters for two bird species, least tern (*Sterna antillarum*) and European kingfisher (*Alcedo atthis*). Overall, this project demonstrated an approach that could be used to quantitatively integrate mechanistic endocrine disruptor research with population biology and life history parameters.

This modeling research (1) supports the identification of safe levels of contaminants by improving the assessment of potential reproductive and survival impacts to wildlife populations, (2) provides information that improves the relevance and accuracy of ecological risk assessments, and (3) supports EPA's ability to objectively and scientifically assess ecological risks and evaluate risk management options.

## Impacts and Outcomes of the Research

As highlighted in the preceding research summaries, the Endocrine Disruptors Research Program made significant progress in understanding sources of EDCs in the environment and EDC effects on wildlife in individual animals and in populations. Impacts and outcomes include the following:

- Evaluated suspected sources of EDCs in the environment and identified chemicals that have the potential to feminize or masculinize freshwater fish. Developed methods to detect estrogenic activity of substances in surface waters and to characterize hormone levels in complex wastewater effluent. The data and methods provide hazard and exposure data necessary for ecological risk assessments of these sources to determine whether additional controls to reduce EDC discharges are warranted. Data will support the identification of treatment practices to reduce impacts of EDC pollution.
- Improved our scientific understanding of baseline endocrine function and effects of EDCs in birds, frogs, fish, crustaceans, and snails. The research illuminated potential mechanisms of endocrine disruption for a range of EDCs including estrogen derivatives, retinoids, tributyltin, organochlorine pesticides, insect juvenile hormone pesticides, and dioxin. These data are critical for characterizing the sensitivity of different wildlife species to EDCs and extrapolating between species. The data improve the accuracy of ecological hazard assessments of EDCs for a set of ecologically important organisms.
- Evaluated exposures to and effects of EDCs occurring in wild populations of birds, sharks, alligators, and crocodiles. These studies determined levels of contamination of animals in the wild and provided indications of whether animals in the wild are more or less sensitive to endocrine disruption compared to laboratory animals.
- Developed tools for translating the effects of EDCs on individual organisms into population-level impacts. These tools help scientists analyze the significance of EDC toxicity or indicators of exposure in terms of ecosystem structure and function, which improves the quality of ecological risk assessments and evaluations of risk management approaches.

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