

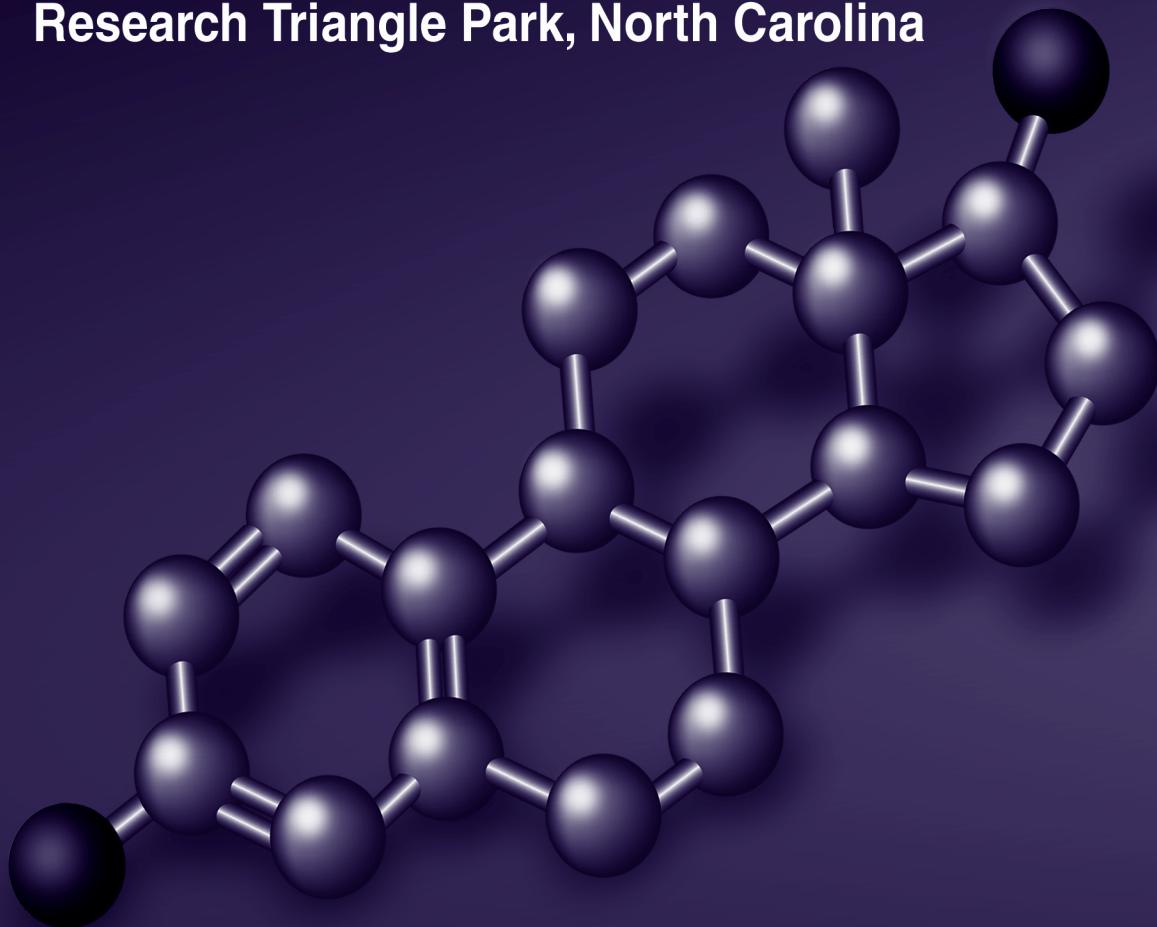


# Workshop Abstracts

## Endocrine Disruptors Program Review Workshop

October 29-31, 2002

Research Triangle Park, North Carolina



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## Introduction

Evidence suggests that environmental exposure to some anthropogenic chemicals may result in disruption of endocrine systems in human and wildlife populations. A number of the classes of chemicals suspected of causing endocrine disruption fall within the purview of the U.S. Environmental Protection Agency's (EPA) mandates to protect both public health and the environment. Although there is a wealth of information regarding endocrine disruptors, many critical scientific uncertainties still remain.

In 1996, EPA's Office of Research and Development (ORD) identified endocrine disruption as one of its top six research priorities and developed a risk-based research approach to address some of these uncertainties. ORD's research program is based on a peer-reviewed Research Plan published in 1998 ([www.epa.gov/ORD/WebPubs/final](http://www.epa.gov/ORD/WebPubs/final)) and has three long-term goals:

- *Providing a better understanding of the science underlying the effects, exposure, assessment, and risk management of endocrine disruptors.* Research in this area includes determining: dose-response relationships, the effects of exposure to multiple endocrine disruptors, major sources of exposure, and approaches for managing risks.
- *Determining the extent of the impact of endocrine disruptors on humans, wildlife, and the environment.* Research includes determining: what effects are occurring in human and wildlife populations, the chemical classes of greatest concern, the ambient levels of exposure, and how unreasonable risks can be mitigated.
- *Supporting EPA's screening and testing program.* ORD is developing needed computational tools as well as *in vitro* and *in vivo* assays in support of the implementation of a screening and testing program for endocrine disruptors, required by the 1996 Food Quality Protection Act.

ORD's intramural research program is conducted across three national laboratories—National Health and Environmental Effects Research Laboratory, National Exposure Research Laboratory, and National Risk Management Research Laboratory—and through one of its two national centers, the National Center for Environmental Assessment. ORD's extramural research program is carried out through its other national center, the National Center for Environmental Research, which is responsible for implementing the Science to Achieve Results (STAR) competitive grants program. EPA's intramural research related to endocrine disruptors has been ongoing for several decades, and was integrated into a coordinated research program in the mid-1990s. Since 1996, EPA has issued four Requests for Applications in the area of endocrine disruptors, two of which have been with other federal agencies and with whom EPA collaborates under the auspices of a Working Group under the Committee on Environment and Natural Resources of the President's National Science and Technology Council.

This Program Review Workshop brings together EPA's intramural and extramural scientists as well as scientists funded by other federal agencies who are working to address the uncertainties associated with exposure and effects of endocrine-disrupting chemicals in the environment. EPA uses Program Reviews such as this one to allow EPA and other federal and non-federal scientists to discuss research progress on topics of major scientific interest to the Agency. The research reported here is of critical importance to EPA, as it has the potential to strengthen the scientific basis for both assessing the risk from exposure to endocrine disruptors and developing appropriate risk-management practices to mitigate their effects.

The abstracts in this report are organized into platform presentations and poster presentations, and are presented in alphabetical order by research category. The research described in this report has not been subjected to the Agency's required peer review and policy review, and does not necessarily reflect the views of the Agency. Therefore, no official endorsement should be inferred. Any opinions, findings, conclusions, or recommendations expressed in this report are those of the investigators who participated in the research and the Program Review Workshop, and not necessarily those of EPA or the other federal agencies supporting the research.

For further information on EPA's Endocrine Disruptors Research Program, please contact the National Program Director, Elaine Z. Francis, Ph.D., by telephone at (202) 564-6789, or by e-mail at [francis.elaine@epa.gov](mailto:francis.elaine@epa.gov).



# **Platform Presentations**

## **Background**

## **Overview of EPA's Regulatory Program**

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The Food Quality Protection Act of 1996 requires the U.S. Environmental Protection Agency (EPA) to develop and implement a screening program to determine whether pesticides may have estrogenic effects in humans. It and other laws provide authority to screen for other endocrine effects, screen other substances that may have an effect cumulative to an effect of a pesticide, examine drinking water contaminants to which a substantial population may be exposed, and screen for effects in wildlife. The program must use “appropriate validated test systems and other scientifically relevant information.”

An Advisory Committee convened by EPA found that there currently were no appropriate validated test systems for endocrine effects. It recommended general criteria that a screening program should meet, and suggested a two-tiered battery of assays which, if validated, could serve as a screening program. The Advisory Committee also suggested a scheme for prioritizing chemicals for screening. EPA's Endocrine Disruptor Screening Program (EDSP)

currently is involved in several activities, most of which are based on the Advisory Committee's recommendations:

- Validation of assays
- Coordination of regulatory considerations for pesticides and other chemicals
- International harmonization of screening programs for endocrine disruption
- Prioritization of chemicals for screening.

Most of the current efforts by the EDSP are directed towards validation of assays and selection of an appropriate battery for use. Validation of assays follows principles set forth by the Interagency Coordinating Committee for the Validation of Alternative Methods, a committee of U.S. government agencies recently established to coordinate the development, validation, acceptance, and harmonization of alternative toxicological test methods throughout the federal government.

## **Screening and Testing Assays**

## Mosquitofish as a Model Organism for the Study of Endocrine-Disrupting Chemicals

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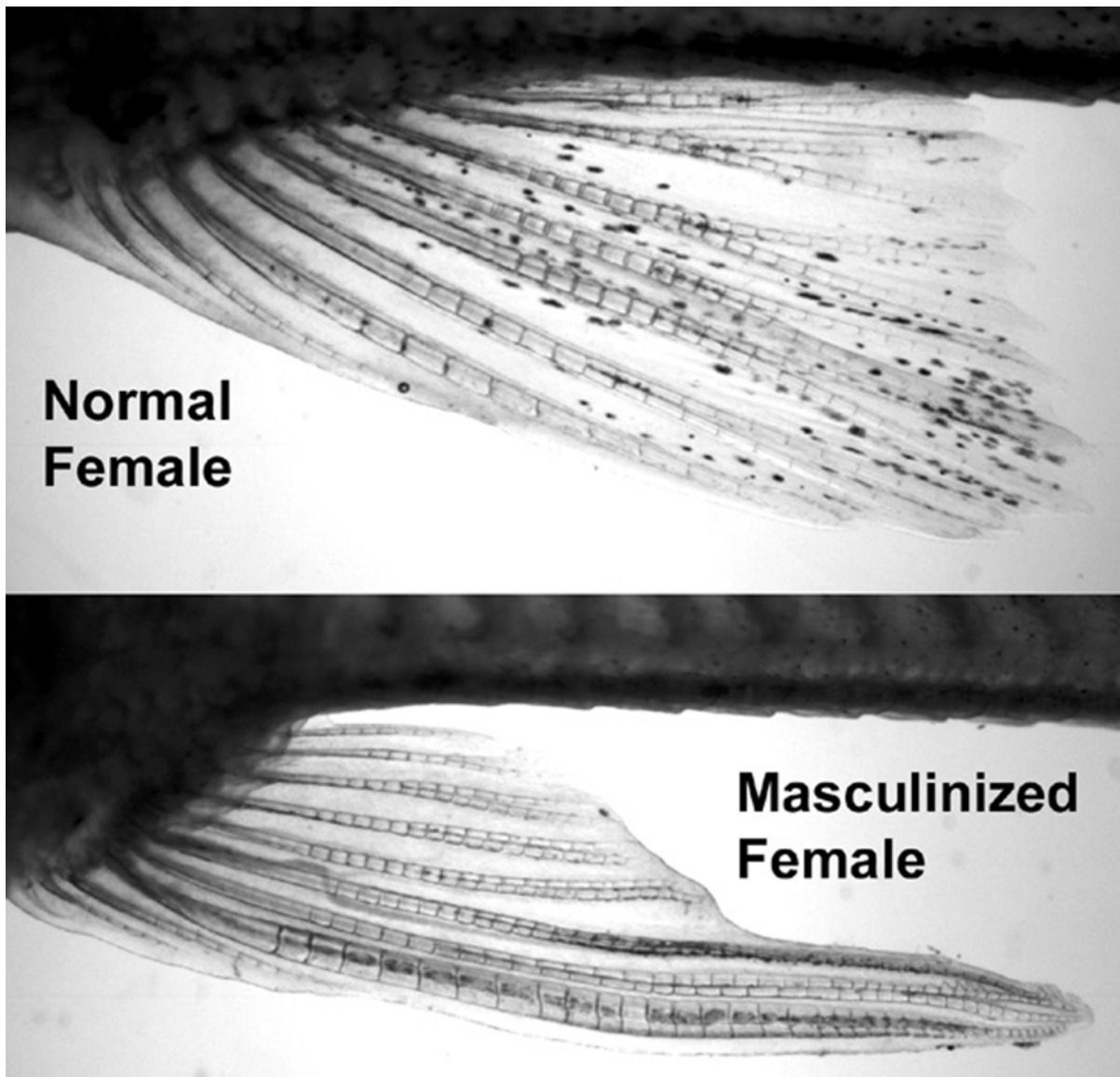
In recent years, evidence has been accumulating that many of the chemicals that humans have introduced into the environment may act as hormones and have the potential to disrupt the endocrine systems of wildlife and humans. This has led to the realization that there is a need for the development and validation of *in vivo* and *in vitro* test methods to screen toxicants for endocrine-disrupting activity in vertebrate and invertebrate species. In response to that need, this research group has conducted the necessary background studies to establish the mosquitofish (*Gambusia affinis*) as a model organism for *in vivo* screening of substances with suspected endocrine activity. This small live-bearing fish in the guppy family is easily kept and bred in aquaria. It is abundant in nature and available from commercial suppliers. Its natural range includes fresh and brackish waters throughout the Southeastern United States. Mosquitofish have been introduced into the Western United States and into areas with moderate climates throughout the world.

Expression of the vitellogenin gene is used to test chemicals for estrogenic activity. Vitellogenin is an egg yolk protein normally only expressed in females in response to estrogen. The presence of vitellogenin in the serum of males is evidence of estrogen exposure. This research group has developed and published a quantitative Western blot assay for mosquitofish vitellogenin for this purpose. As part of these studies, the extent to which the vitellogenic response to estrogen exposure is temperature-dependent has been characterized.

A morphological trait is used to test for androgens. Male mosquitofish have a highly elongated and modified anal fin (gonopodium) not found in normal females. If a female is exposed to an androgen, her anal fin will be induced to develop into a gonopodium-like structure (see Figure 1). The presence of modified anal fins in female mosquitofish is visible evidence of exposure to an androgenic substance. This research group exposed female mosquitofish to a variety of known androgens and characterized the response using digital photography and computer image analysis techniques.

As part of this project, steroids have been isolated and identified from the water of a river receiving paper mill effluent where female mosquitofish are masculinized. Mosquitofish are now being used in laboratory exposure studies to study the effects of those chemicals on reproductive development and function.

In addition to their suitability as laboratory test organisms, mosquitofish are useful as sentinel species for the detection of endocrine disruptors in aquatic environments. Field studies of mosquitofish have included evaluations of reproductive anatomy and fitness in: (1) a population in which females have been masculinized by exposure to androgens in paper mill effluent, and (2) a population living below the outfall of metropolitan wastewater treatment plants to investigate possible evidence of exposure to estrogens.



**Figure 1.** The anal fins of female mosquitofish exposed to an androgen are induced to develop into gonopodium-like structures.

## Identification of Endocrine Disruptors Using a Short-Term Reproduction Assay With the Fathead Minnow

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Tests with small fish have been a recommended component of virtually every proposed regulatory program for endocrine-disrupting chemicals (EDCs). An ideal test would provide information suitable for determination of whether a chemical has the potential to exert adverse effects via specific modes/mechanisms of action (MOA) of concern, as well as supply dose-response data useful for higher tier risk assessments (e.g., growth, reproduction). To address these needs, a short-term reproduction assay was developed utilizing reproductively mature fathead minnows (*Pimephales promelas*). Endpoints evaluated in the assay include those specific for endocrine-related MOA

(e.g., plasma vitellogenin and steroid concentrations), and those reflective of general reproductive fitness (e.g., fecundity, fertility, F1 viability). The test has been evaluated using model EDCs with suspected/known MOA, including strong and weak estrogen receptor agonists (estradiol, methoxychlor), androgen receptor agonists (methyltestosterone, trenbolone), androgen receptor antagonists (vinclozolin, flutamide), and inhibitors of steroid metabolism (fadrozole). The test has consistently and accurately characterized the different chemicals both with respect to their reproductive toxicity and through alterations in one or more endpoints reflective of presumed MOA.

## Environmentally Mediated Endocrine Disruption in Estuarine Crustaceans: Meiobenthos-Based Life-Cycle Assays for Evaluating Risks of Reproductive and Endocrine Toxicity

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In the majority of the world's estuaries, persistent contaminants of potential endocrine and reproductive toxicity reside in sediments almost continuously at nonlethal exposure concentrations. Endocrine-disrupting chemicals (EDCs) can mimic natural hormone action by either agonizing/antagonizing hormonal effects, modifying hormone receptor structure, or recognizing/blocking hormonal binding sites. For example, ecdysteroids are known to be important regulators of molting, embryonic development, metamorphosis, reproduction, and pigmentation in insects, crustaceans, and even nematode worms; thus, the endocrine-disrupting bisacylhydrazine insecticides exert control by targeting ecdysteroid receptors.

Vitellogenesis—the process of yolk synthesis—is hormonally regulated and potentially altered by EDCs that interfere with proper ecdysteroid action. Thus, the health and ultimate quality of developing crustacean embryos is absolutely dependent on ecdysteroid balance and the concentration and nutritional quality of vitellin—the major lipoprotein produced via vitellogenesis. During the past 3 years, and from the Environmental Protection Agency's (EPA) Science to Achieve Results Program support, meiobenthos-based sediment ecotoxicology has moved away from “kill ‘em and count ‘em” approaches to more sophisticated life-cycle approaches targeting biochemical and reproductive effects directly linked to population maintenance (e.g., genetic change, fertilization success, altered sex ratios, masculinization/feminization, vitellogenesis, egg quality, reproduction, recruitment, and population growth modeling) under the mid- to low-level chronic exposures typical of urbanized settings.

For the meiobenthos and most invertebrates, the majority of life-cycle screening tools have not been available for assessing the endocrine/reproductive disrupting potentials of contaminants. Therefore, this research project built on

prior meiobenthos culturing technologies to develop: (1) a microplate culturing assay with the benthic copepod *Amphiascus tenuiremis* in which 18-hour old nauplii (or stage 1 copepodites) are reared individually to reproductive maturity under EDC exposure in hydrophilic (low binding) 96-well microtiter plates for 10-12 days and then mated; (2) a spiked-sediment microassay in which nauplii or stage 1 copepodites are cultured to F<sub>1</sub> production under continuous EDC exposure; (3) an *in vivo* lipovitellin-based semiquantitative assay of copepod individual egg/embryo quality using dual-channel laser-scanning confocal microscopy (LSCM); (4) two new enzyme-linked immunosorbent assays (ELISAs) for individual copepod ecdysone and vitellin; and (5) detection of pesticide-induced genetic change.

Eggs/embryos of *A. tenuiremis* are small (approximately 50 µm thick) with a clear chorion and yolk vitellin tightly packaged into vesicles that are easily quantified by LSCM in a nondestructive manner. In microplate assays, individually exposed nauplii (or C-I copepodites) can be monitored through to the copepodite and adult stages via inverted microscope or LSCM for developmental/reproductive effects. ELISAs allow assessment of specific endocrine effects most important to population growth and maintenance. This project focuses on how these approaches have been used to evaluate reproductive/endocrine effects of a “modern” gamma-aminobutyric acid-disrupting cyclodiene insecticide, fipronil. Fipronil induces developmental delays and strong, but reversible, sex-specific reproductive failures in copepods at environmentally realistic concentrations. Fipronil is receiving rapidly increased use in the Southeastern United States in crawfish/rice-culture, turf grass management, and fire ant/termite control. In addition to toxicological data and test methods, this project is providing EPA with pesticide fate, degradation, and transport information via mesocosm simulations with laboratory-based salt-marsh ecosystems.



## Complementary *In Vitro* and *In Vivo* Rodent Assays

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In attempts to identify pollutants, drugs, dietary constituents, and other compounds possessing hormone agonist or antagonist activity, *in vitro* assays offer many advantages, including relative simplicity, rapidity, and low cost. However, only *in vivo* assays provide the pharmacokinetic and pharmacodynamic context necessary to much more fully predict the compound's effects on humans or other organisms of concern. An appealing strategy, therefore, involves an initial *in vitro* screening to identify a subset of compounds for which *in vivo* testing is warranted.

In a first phase of studies aimed at identifying and characterizing pollutant (anti)estrogens, both cell-free competitive estrogen receptor (ER) binding assays and ER-transactivation reporter gene assays in human breast cancer cells were performed *in vitro* on a set of related com-

pounds. For parent compounds inducing transactivation but not receptor binding, key metabolites also were examined to verify that the metabolites responsible for activity are those that are formed *in vivo* by the species of interest. Using these initial data, one compound, benzo[a]pyrene, was selected for *in vivo* testing using the mouse uterotrophic assay. When both benzo[a]pyrene and major metabolites were unable to induce either uterine weight or expression of lactoferrin, highly estrogen-inducible endpoints in the uterus, a microarray strategy was adopted. These microarrays, enriched in estrogen-regulated genes, do not depend on increases in organ weight and therefore can be used on any tissue of interest. This ongoing work will be particularly useful for the detection and characterization of compounds that interact with the ER only in a subset of tissues and that, therefore, may be missed in traditional, solely *in vitro* and uterine-based testing.

## Endocrine Disruptors—Tiered Screening and Testing: Filling Key Data Gaps

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The U.S. Environmental Protection Agency (EPA) is developing a screening and testing program for endocrine-disrupting chemicals (EDCs). High-priority chemicals would be evaluated in the Tier 1 Screening (T1S) Battery. Chemicals determined to be positive in T1S then would be tested (Tier 2). T1S includes *in vitro* estrogen receptor (ER) and androgen receptor (AR) binding and/or gene expression, an assessment of steroidogenesis, and mammalian and nonmammalian *in vivo* assays.

Using the rat, the uterotrophic assay detects estrogens and antiestrogens, while steroidogenesis, estrogenicity, and hypothalamic-pituitary-gonadal function are assessed in a “Pubertal Female Assay.” (Anti-)androgens are detected in the Hershberger Assay (androgen-dependent tissues in castrate-immature male rats). Fish and amphibian assays also are being developed. The fathead minnow assay can identify EDCs displaying several mechanisms of concern, including AR and ER, AR antagonists, and aromatase inhibitors, but the amphibian assay is designed to detect thyroid-active substances. Several alternative mammalian

*in vivo* assays have been proposed. Of these, a short-term pubertal male rat assay appears most promising. An *in utero*-lactational screening protocol also is being evaluated. For Tier 2, the numbers of endocrine-sensitive endpoints and offspring (F1) examined needs to be expanded.

Consideration also should be given to tailoring T2, based on the results of T1. For example, endpoints such as anogenital distance at birth and nipple/areola retention in infant rats should be required in testing for androgens and antiandrogens because they are sensitive, permanent effects that are highly correlated with malformations and reproductive organ weight changes later in life. EDCs that display antithyroid, estrogenic, or antiestrogenic activity display a different profile of developmental effects, and these aforementioned endpoints are not sensitive to disruption by these mechanisms. Tiers 1 and 2 also should examine relevant mixtures of EDCs. For example, toxicants that induce malformations in androgen-dependent tissues produce cumulative effects, even when two chemicals act via different mechanisms of action.

## The Medaka: An *In Vivo* Model for Detection of Adverse Effects of Endocrine Disruptors

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The principal objective of this research is to develop and validate the use of a short-term *in vivo* model—the small fish, medaka (*Oryzias latipes*)—to identify adverse effects of exposure to endocrine-disrupting chemicals. It is possible to determine sex without invasive methodology using secondary characteristics or by detection of phenotypic markers in mutant species. Sex reversal follows exposure to estrogenic or androgenic hormones. Calibration of breeding pairs prior to exposure improves the ability to detect reproductive impairment. The small body size, coupled with a newly developed, transparent medaka, diminishes cost and improves whole-body survey histopathology while extending phenotypic detection from embryonic to adult life stages. The compressed life cycle of the medaka makes it possible to perform multigenerational assays in a reasonable amount of time.

These studies are designed to integrate findings across levels of biological organization. Molecular and biochemical studies have addressed the role of genes associated with steroid biosynthesis and metabolism in endocrine

disruption. Multiple forms of cytochrome P450, critical for metabolism of numerous endobiotics including testosterone, estrogen, and retinoic acid, have been isolated and characterized. These studies also have addressed the promotion of tumorigenesis by endogenous estrogen and xenobiotics with hormone-like activity. Cellular and tissue analyses first characterized the effects of endocrine-disrupting compounds on active, quiescent, and recrudescing gonads. Results from these studies demonstrated atresia of oocytes, supportive cells, stromal alteration, and focal necrosis (testis). At the individual level, the most sensitive developmental stage for adverse gonadal and/or reproductive effects followed initiation of exposure immediately after hatching. At the population level, using rigidly controlled laboratory conditions, exposure to mixtures of various endocrine-disrupting compounds at environmentally relevant concentrations reduced reproductive success. The use of medaka as a model for evaluating effects of endocrine disruption at all levels of biological organization will continue to be explored.

## Validation of an *In Vivo* Protocol To Evaluate the Effects of Environmental Chemicals on Female Pubertal Development and Thyroid Function

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In response to emerging concerns that environmental chemicals may have adverse effects on human health by altering the function of the endocrine system (<http://www.who.int/pcs/>), the Food Quality Protection Act mandated that the U.S. Environmental Protection Agency (EPA) develop and implement an endocrine disruptor screening program (EDSP). Working toward this goal, EPA currently is implementing a proposed EDSP that is designed to detect chemicals that alter the estrogen, androgen, and thyroid systems in humans, fish, and wildlife (<http://www.epa.gov/scipoly/oscpendo/index.htm>). Studies currently are ongoing within the Agency to develop, standardize, and validate a number of *in vitro* and *in vivo* mammalian and ecotoxicological assays for use in a Tier 1 Screening Battery.

This project focuses on studies that support the validation of the *in vivo* protocol to evaluate the effects of environmental chemicals on female pubertal development and thyroid function in the juvenile rat. Using a 20-day dosing regimen that encompasses the critical period of sexual maturation for the female rat, this protocol has the ability to detect agents that display antithyroid, estrogenic,

antiestrogenic (estrogen receptor or steroid-enzyme mediated) activity, or alter puberty via changes in luteinizing hormone, follicle-stimulating hormone, prolactin and growth hormone secretion, or via alterations in hypothalamic function. Initial studies have identified the more sensitive endpoints, provided an assessment of the robustness of the protocols with regard to inter- and intra-laboratory and inter-strain sources of variation, and standardized operating procedures. Subsequent studies have assessed the reliability (e.g., ability to be replicated in multiple laboratories) and the relevance of the protocol (e.g., ability to provide a measure of a specific biological process) using chemicals with known mechanisms of action. Specific issues pertaining to study design that could possibly confound the results have been evaluated. For example, a study to assess the relationship between growth rate and pubertal development demonstrated that all endpoints were unaffected, even in the presence of a 10 percent reduction in body weight, a limit set for dose selection based on the maximum tolerated dose. In summary, these studies demonstrate that the female pubertal protocol is a reliable and fairly simple screen to detect endocrine-disrupting chemicals with multiple mechanisms of action.

## Reproductive and Developmental Screening Protocols for Endocrine Disruptors Using Estuarine Crustaceans

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The objective of this research is to develop *in vivo* screening protocols for endocrine disruption in marine crustaceans, invertebrates of ecological and economic importance. A series of comparative developmental and reproductive studies were performed on several species of estuarine crustaceans in response to three juvenile hormone agonists (JHAs) (methoprene, fenoxycarb, and pyriproxyfen). Larval development of the grass shrimp, *Palaemonetes pugio*, was greater than two orders of magnitude more sensitive to disruption by JHAs than was embryonic development. Fenoxycarb-exposed larvae had significantly altered levels of ecdysone, the hormone that along with juvenoids is known to regulate the metamorphic process in decapod crustaceans. For two of the three JHAs under similar static exposure conditions, developing larvae of the xanthid mud crab, *Rhithropanopeus harrisi*, exhibited reduced metamorphic success at lower concentrations than grass shrimp larvae.

These comparative responses suggest that the more rigidly controlled metamorphic process in crabs is more sensitive to compounds acting as endocrine disruptors than is the more plastic metamorphic pattern seen in shrimp. The final crab larval stage, the megalopa, was more sensitive to JHA exposure than earlier zoeal stages. Mud crab larvae exposed to fenoxycarb had reduced bio-

mass and lipid content, particularly triglycerides and free sterols, at concentrations below which inhibited metamorphic success. Concentrations of fenoxycarb, which reduced the reproductive capacity in single life-cycle exposures of the estuarine mysid, *Americamysis bahia*, were similar to those concentrations that inhibited metamorphosis in grass shrimp under similar flow-through exposure conditions. Juvenile mysids were released by exposed adults and reared through maturation without further exposure; however, they produced fewer young and had altered sex ratios (reduced percentages of males) at lower parental-exposure concentrations than directly impacted parental reproduction.

Because the endocrine glands responsible for regulating mysid sexual differentiation and reproduction develop during larval stages, these transgenerational responses may well be a product of irreversible effects during developmental exposures that become apparent following maturation and initiation of reproduction. These findings suggest the necessity of at least a two-generational mysid exposure protocol for adequately predicting the ecological risk of chemicals acting as endocrine disruptors on crustaceans that function as the dominant secondary producers in estuarine ecosystems.

## Thyroid Axis Inhibition in *Xenopus laevis*: Development of an Amphibian-Based Screening Assay for Thyroid Disruption

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In response to the initial Endocrine Disruptor Screening and Testing Advisory Committee recommendations, research was conducted on the development of a *Xenopus laevis*-based tail resorption assay for evaluating thyroid axis disruption. These experiments highlighted key limitations that are associated with relying on tail resorption as a measure of anti/thyroid activity. The most critical limitation is that tail tissue of tadpoles in metamorphic climax are insensitive to perturbation by agonists/antagonists. To improve on the initial proposal, this research group conducted experiments comparing the sensitivity of premetamorphic (stage 51) and prometamorphic (stage 54) tadpoles to the model thyroid axis antagonists methimazole (control, 6.25, 12.5, 25, 50, and 100 mg/L); 6-propylthiouracil (control, 1.25, 2.5, 5, 10, and 20 mg/L); perchlorate (control, 15.6, 62.5, 250, 1,000, and 4,000 µg/L); and iopanoic acid (control, 23.4, 93.8, 375, 1,500, and 6,000 µg/L). Tadpoles were exposed for a 2-week period, and developmental stage,

thyroid size, and histology were examined at 1 and 2 weeks after exposure.

Methimazole, 6-propylthiouracil, and perchlorate, which are thyroid hormone synthesis inhibitors, all caused a concentration-dependent inhibition of the thyroid axis. Further, these three compounds caused dose-dependent changes in thyroid gland morphology. These changes were characterized as reduced colloid, glandular hypertrophy, and cellular hyperplasia. Treatment failed to affect growth, even in tadpoles that experienced significant metamorphic inhibition. As determined from these endpoints, there were only minor differences in sensitivity observed between the two stages examined. Iopanoic acid, a type II deiodinase inhibitor, surprisingly stimulated metamorphosis. These results indicate that tadpoles in the early stages of metamorphosis are sensitive to thyroid axis inhibition and that development of a short-term, diagnostic amphibian-based thyroid screening assay shows considerable promise.

## Novel Cheminformatics and Pattern Recognition Tools Useful for Risk Assessment and Regulatory Control

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Computer-aided molecular modeling (CAMM), originally developed for rational drug design, has demonstrated its utility in a wider range of applications, including environmental risk assessment and computational toxicology. Approaches in CAMM can be reduced to two general strategies: (1) receptor-based modeling, and (2) ligand-based modeling. The former case refers to the situation in which the three-dimensional (3D) structure of the target receptor is available, thus enabling one to exploit the familiar “lock and key” paradigm. The latter case refers to the all-too-frequent situation in which the receptor’s 3D structure is not known, thus requiring one to discern the relationship between molecular structure and biological activity by inferring information embedded in pertinent structure-activity data. In ligand-based approaches, the transformation from data to knowledge and insight is greatly facilitated by the construction of quantitative structure-activity relationship (QSAR) models. Basically, QSAR models employ statistical regression techniques to correlate variations in the biological activity of a series of chemicals with variations in their molecular structure as encoded in calculated structural features and properties (descriptors). In risk assessment, QSAR models can serve as powerful tools to screen large chemical databases in search of potentially hazardous agents, predict the biological activity of these agents prior to biological testing, and gain insight into their

mechanism of action and structural prerequisites for biological activity.

To illustrate the utility of both receptor-based and ligand-based approaches including QSAR models in risk assessment, examples will be taken from ongoing research projects in the Welsh Laboratory. The presentation will conclude by introducing several new computational tools that show great promise for use in many areas, including risk assessment and computational toxicology. For example, the Polynomial Neural Network (PNN) is a powerful iterative neural network algorithm that automatically produces QSAR models in parametric form [ $Y = f(X_i)^n$ ], where the  $X_i$  terms can be linear ( $n = 1$ ) or nonlinear ( $n = 2, 3$ , etc.). The PNN thus combines the best features of artificial neural networks (i.e., inherent nonlinearity) and multivariate regression analysis (i.e., analytical equation) into a single entity. The PNN also is remarkably insensitive to outliers and “noisy” data that often confound normal regression methods. Second, the “Shape Signatures” algorithm employs a unique concept based on molecular shape and electrostatic properties that enables rapid screening of large numbers of small molecules against each other or even against a receptor pocket. The utility of the PNN and Shape Signatures will be demonstrated using examples associated with the identification and prediction of endocrine-disrupting compounds.

## **Developmental Exposures**



## Short-Term Exposure to an Environmental Mixture of PHAHs: Dose-Additive Effects on Serum Thyroxine

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Endocrine disruption from environmental contaminants has been linked to a broad spectrum of adverse outcomes. An additional concern about endocrine-disrupting xenobiotics is the potential for additive or synergistic effects of mixtures. A short-term dosing model to examine the effects of environmental mixtures on thyroid homeostasis (TH) has been developed. Prototypic chemicals such as dioxins, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers have been shown to alter TH homeostasis in this model primarily by upregulating hepatic catabolism of thyroid hormones. Current efforts are testing the hypothesis that the effects of mixtures of these chemicals on thyroxine (T4) concentrations can be predicted by dose-additivity theory. The current study used 28 day-old female Long-Evans rats, orally dosed with varying concentrations of 18 different planar halogenated aromatic hydrocarbons (2 dioxins, 4 dibenzofurans, and 12 PCBs, including dioxin-like and non-dioxin-like PCBs) for

4 consecutive days. Serum samples were collected 24 hours after the last dose. Serum total T4 was measured via radioimmunoassay. Extensive (7-9 doses/chemical) dose-response functions were statistically modeled to determine median effective doses using the Hill Equation. A mixture was custom synthesized with the ratio of chemicals based on environmental concentrations (e.g., relative to tetrachlorodibenzodioxin: tetrachlorodibenzofuran - 1.5x, PCB 126 - 50x; PCB 153 - 30,000x). Serial dilutions of this mixture were tested in the 4-day dosing assay. Predicted outcome, based on the assumption of dose-additivity, was tested using statistical dose-response modeling. Preliminary analyses of results suggest that the effects of the mixture on serum total T4 can be predicted by dose-additivity. There was no evidence of synergism or antagonism. Future work will expand the mixture to include chemicals from diverse classes of thyroid disruptors such as TH synthesis inhibitors.

## Effects of Endocrine-Disrupting Compounds on Mammary Tissue Development

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Breast cancer risk in women is known to be significantly influenced by genetics and by prolonged exposure to estrogen. However, when all known risk factors and characteristics are considered, more than 50 percent of breast cancer cases remain unexplained. There is a growing body of evidence indicating that exposures to certain toxic chemicals and hormone-mimicking compounds may contribute to the development of breast cancer. Several endocrine-disrupting compounds (EDCs) that act in an estrogenic or anti-androgenic manner are known to alter rodent mammary gland development. Although epigenetic in nature, these xenobiotics may hasten development of the gland and increase the incidence of mammary tumors if they significantly alter serum estradiol levels, or if they change receptor expression patterns, hormone transport, or metabolism that results in altered response to endogenous estradiol levels. Nonylphenol is an example of an EDC that this research group has shown to hasten mammary gland development following acute *in utero* exposure.

Delayed development of the mammary gland also can be caused by *in utero* exposure to EDCs, resulting in im-

printing, or irreversible effect in the offspring. This type of delayed glandular development could lead to increased tumor formation due to a shift or enlargement in the window of sensitivity to carcinogens. For example, a toxicant may delay mammary development so that undifferentiated or dividing cells may be present for longer periods of time, thus rendering the tissue more vulnerable to a subsequent genotoxic insult. The herbicide atrazine and the polyaromatic hydrocarbon dioxin are examples of compounds that delay mammary gland development and increase the potential for mammary carcinogenesis. A delay in mammary gland development has been detected as early as postnatal day 4 and persists throughout puberty in female rats prenatally exposed to atrazine. Similarly, dioxin exposure on gestation day 15 causes an irreversible modification in epithelial migration and branching patterns. Because of altered epithelial differentiation, terminal end buds are present for a longer period of time. These multilayered structures are sensitive to carcinogens, such as 7,12-dimethylbenz[a]anthracene, and exposure to such agents during this critical window of susceptibility could lead to increased multiplicity or decreased latency to tumor formation.

## Analysis of Sensitive Developmental Stages in Birds to EDCs

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Estrogenic exposure of embryonic Japanese quail (JQ), newly hatched zebra finch (ZF), or JQ chicks produced developmental abnormalities of the reproductive tracts, brain, and behavior. Diethylstilbestrol and estradiol benzoate (E<sub>2</sub>B) produced dose-responsive changes of testicular feminization when injected into eggs early in incubation, at doses as low as 10 nmol/g E<sub>2</sub>B. Oral dosing of ZF chicks on days 5-11 after hatching with 10-1,000 nmol/g body wt/day E<sub>2</sub>B produced a dose response in altered courtship and singing behaviors in both males and females, expressed when the birds became adults. Female finches developed the ability to sing, and male finches exhibited reduced courtship and increased nesting behaviors. The xenobiotic compounds dicofol, methoxychlor, and endosulfan had little effect on brain morphology or behavior.

E<sub>2</sub>B treatment at doses of 1-1,000 nmol/g body wt/day altered sexually dimorphic brain nuclei with an increase in the nuclear volume of area X. The three highest doses (10, 100, and 1,000 nmol/g) significantly increased the nuclear area of RA and HVC. Courtship and reproductive behaviors were altered by postnatal estrogenic exposure. The 100 nmol/g and 1,000 nmol/g E<sub>2</sub>B, and 100 nmol/g octylphenol (OP) dosed males displayed lower female-directed song. The 1,000 nmol/g methoxychlor-

dosed males sang more in tests against females. The 10 nmol/g and 100 nmol/g E<sub>2</sub>B females had significantly higher incidences of singing. Females in the 100 nmol/g E<sub>2</sub>B, 1,000 nmol/g E<sub>2</sub>B, and 1,000 nmol/g OP treatment groups exhibited masculine behaviors without singing.

Reproductive testing was conducted either in individual pair cages or in communal cages that permitted self-selection of mates, N = 5-10 pairs per group. Pairs consisted of E<sub>2</sub>B-treated males and females, E<sub>2</sub>B-treated males paired with canola-treated females, vice versa, and canola-treated males and females. Posthatch E<sub>2</sub>B treatment produced sex-specific impairments in reproduction that, in some instances, were additive when both sexes were treated. Egg production was reduced and egg breakage was increased in 100 nmol/g E<sub>2</sub>B-treated male and female pairs. The incidence of missing eggs was increased in 10 nmol/g E<sub>2</sub>B-treated male and female pairs. Candedled fertility was reduced in both groups containing 100 nmol/g E<sub>2</sub>B-treated males. The number of hatched chicks was severely reduced in all E<sub>2</sub>B-treated groups. These significant treatment effects (all p < 0.05) show that posthatch E<sub>2</sub>B treatment profoundly disrupts the reproductive performance of zebra finches, suggesting that exposure to estrogens in the wild could impair the reproductive performance of wild populations.

## Endocrine Disruption in Pubertal Rhesus Monkeys: Growth and Sexual Maturation

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During puberty, a number of systems (reproductive, central nervous, skeletal, immune) complete their final stage of maturation under the influence of estrogen. Monkeys, like humans, undergo what some consider to be a unique period of development during adolescence, which makes them important models for understanding exogenous estrogen effects during this period. This study evaluated a cohort of pubertal female monkeys treated with exogenous estrogen; this report presents data on growth and sexual maturation.

Female adolescent rhesus monkeys (*M. mulatta*) (n = 8/group) received daily oral doses of methoxychlor (MXC; 25 or 50 mg/kg/day), diethylstilbestrol (DES; 0.5 mg/kg/day), or vehicle control for 6 months preceding and following the expected age at menarche (30 months) with a subsequent 8-month recovery period. Serum exogenous (nonsteroidal) estrogen activity as determined by an ER $\alpha$  transcription activation assay was 0, 34, 42, and 60 pg/mL in the control, MXC25, MXC50, and DES groups at the end of the treatment period.

The DES group did not gain weight during treatment and did not recover fully. Height growth also was clearly depressed in the DES group. They weighed 15 percent less than controls and were 8 percent shorter at the end of the study. Although the DES group gained very little weight, they did demonstrate a very attenuated “growth spurt” at the same time as the other monkeys. The MXC groups lagged behind controls in weight

gain only briefly during the growth spurt. Analysis also demonstrated a smaller increase in height in the MXC25 as well as the DES group during the treatment period. Muscle mass, skinfold measures, and body mass index did not differ by group, indicating symmetrical growth retardation. The reduced weight gain in the DES group was associated with both reduced food intake and reduced food use efficiency.

Menarche (first occurrence of vaginal bleeding) occurred 6 months later in the DES group than in controls. Although MXC groups were not significantly delayed, regressions indicated that higher serum exogenous estrogen was associated with later first menses in the cohort as a whole. The DES group menses very infrequently during treatment (average 1 day in 6 months), but all began menses shortly (average 5 $\pm$ 1 days) after treatment was discontinued, suggesting that menses was suppressed rather than menarche delayed. Both DES and MXC led to premature emergence of a secondary sex characteristic, reddening and swelling of skin, but retarded growth of the nipple also was observed. Uterine size and endometrial thickness, measured by ultrasound after the recovery period, were not influenced by treatment.

In conclusion, exogenous estrogens did not accelerate pubertal growth and development, but generally retarded them. Most of the effects on growth and morphology were reversible, but functional consequences have not been evaluated.

## The Effects of *In Utero* and Lactational Exposure to Genistein or Daidzein on Reproductive Development in FVB/N Mice and on Occurrence of Mammary Tumors in MMTV-*neu* Transgenic Mice

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Exposure to estrogens during critical windows of development may affect reproductive and mammary development. In this study, researchers determined whether *in utero* or lactational exposure to the phytoestrogens genistein and daidzein affect reproductive development and occurrence of mammary tumors in mice. For the reproductive studies, FVB/N dams were treated with diethylstilbestrol (DES; 0.03 mg/kg/day), daidzein (40 mg/kg/day), or genistein (4 and 40 mg/kg/day) during pregnancy (gestational day 14-18) and/or lactation (birth-weaning). The dams and the offspring were fed a semipurified (isoflavone-free) diet, and all treatments were given to the dams by gavage to prevent direct exposure of the pups. Endpoints measured at birth, weaning, and/or sexual maturity (2 months of age) in the FVB/N offspring included anogenital distance, body weight, uterine weight, testes weight, onset of puberty, and estrus cycling. As expected, the potent estrogen DES altered many reproductive outcomes in both male and female mice. For some of these endpoints, daidzein and genistein mimicked DES; however, unique or opposite responses also were evident for each of the phytoestrogens, including the low dose of genistein. Changes were evident for both male and female pups with *in utero*, lactational, and both exposures to all four treatments compared to the appropriate control group. The window of exposure also was important because the *in utero* and lactational exposures often had different effects. Adult outcomes such as estrous cycling and uterine or testicular weights showed that treat-

ment effects were evident well after the end of the exposure window (birth or weaning). These data indicate that *in utero* and lactational exposure to isoflavones can influence reproductive development of mice.

For mammary tumor studies, wild-type FVB/N dams were mated with MMTV-*neu* transgenic males, yielding *c-neu* hemizygous offspring. Mammary tumors spontaneously arise in this model due to activation of the *neu* proto-oncogene. These dams were treated with daidzein (40 mg/kg/day) or genistein (40 mg/kg/day) during pregnancy (gestational day 14-18) or lactation (birth-weaning), and their female offspring were monitored for tumor development until 13 months of age. Neither genistein nor daidzein affected mammary tumor latency or number of mammary tumors per female via *in utero* or lactational exposure. Although lactational exposure to daidzein increased the percentage of females that remained tumor free at maximum age (13 months of age), *in utero* exposure to daidzein or lactational exposure to genistein reduced the fraction of tumor-free females. In the MMTV-*neu* model, in which tumors are induced by the most commonly overexpressed oncogene in human breast cancer, there appears to be no protective effect resulting from developmental exposure to these isoflavones, as has been reported for carcinogen-induced tumors in other animal models. Assessments of rates of metastases and further analyses of tissues from these animals are underway.

## The Michigan PBB Cohort 20 Years Later: Endocrine Disruption?

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In 1973, inadvertent substitution of a livestock feed supplement with fire retardant led to widespread contamination of meat and dairy products in Michigan with polybrominated biphenyls (PBBs), a class of chemicals toxicologically similar to polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), and dioxin, that are suspected of disrupting endocrine function in humans and wildlife. More than 4,000 individuals with high likelihood of exposure were subsequently enrolled into the Michigan PBB Cohort, and serum samples were analyzed for PBB.

Research on the health of exposed women and their daughters has revealed a number of interesting findings. Daughters of highly exposed women who were exposed *in utero* and through breastfeeding had an earlier age at menarche than unexposed girls. PBB (as well as DDT, PCBs, and dioxins) are concentrated in fatty tissue and breast milk. These girls may have been exposed during a critical stage in development of their endocrine and reproductive systems. Preliminary analyses also reveal an extremely high rate of miscarriage among highly exposed daughters who have entered their reproductive years.

PBB exposure among adult women was not associated with duration of lactation, benign breast disease, uterine fibroids, hip fractures, or age at menopause. There was some evidence of an association of PBB exposure with altered menstrual function among highly exposed women who had experienced recent weight loss. It is possible that recent weight loss mobilized the PBB from fat stores, thereby causing an increase in circulating PBBs.

Future research on this cohort should examine the reproductive health of male members of the cohort and sons of exposed parents. Followup of the important findings among women should include detailed study of pubertal development, menstrual function, and early pregnancy.

This cohort provides an important opportunity to determine the human reproductive effects of endocrine-disrupting chemicals. Because of the large number of individuals with documented exposure *in utero* and through breastfeeding, this cohort represents a rare opportunity to study exposure during critical periods of development. During these critical periods, the developing fetus and infant are more susceptible to the disrupting effects of chemical exposures.

## Effects of Trihalomethanes on Pregnancy Maintenance in Rats

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In a recent epidemiological study<sup>1</sup>, consumption of high concentrations of trihalomethanes (THMs), particularly bromodichloromethane (BDCM), were associated with an increased risk of spontaneous abortions. This research group has shown that bromoform and BDCM, two of the brominated THMs, cause pregnancy loss (i.e., full-litter resorption) in F344 rats. In view of the concerns raised by the epidemiological data, BDCM-induced pregnancy loss is being investigated to determine its mode of action in rats. This investigation involves several facets of experimentation: (1) determination of the critical period of pregnancy sensitive to the effect, (2) evaluation of hormonal profiles, (3) hormonal replacement to rescue the pregnancy, (4) *ex vivo* evaluation of critical tissues, and (5) strain comparisons.

In early work, it was demonstrated that the critical period for BDCM-induced full-litter resorption is during the luteinizing hormone (LH) dependent period of pregnancy (gestation days 7-10). During this period, LH is required to maintain luteal secretion of progesterone that, in turn, is required to maintain pregnancy. In an evaluation of hormonal profiles, it was demonstrated that pregnancy loss is indeed associated with decreases in serum LH as well as progesterone. Furthermore, exogenous progesterone and human chorionic gonadotropin (hCG), an LH agonist, were effective in rescuing BDCM-exposed pregnancies. These findings strongly support the hypothesis that BDCM's mode of action is mediated by a disruption of pituitary LH secretion. Exogenous GnRH increased LH levels, suggesting that BDCM's effect is

mediated by GnRH, rather than nonresponsiveness of the pituitary.

In view of the epidemiological association of BDCM and spontaneous abortion, it is important to consider the possibility that BDCM may disrupt pregnancy maintenance by acting at multiple target sites. It remains unclear whether BDCM's effect on pituitary LH secretion in rats may be analogous to a potential effect on placental hCG secretion in humans. Further, because hCG and LH bind to the same luteal receptor, it is important to consider the hypothesis that BDCM also may decrease luteal responsiveness to LH/hCG. To test this hypothesis, rat corpora lutea (CL) were examined *ex vivo* for their ability to secrete progesterone following stimulation with hCG. BDCM had no effect on luteal responsiveness to hCG; however a "rebound" increase in *ex vivo* progesterone secretion was evident in CL previously exposed to BDCM *in vivo*. This finding was unexpected in view of the reduced serum progesterone levels observed following BDCM exposure. It is unclear if this rebound effect may reflect the removal of the CL from a possible inhibitory influence of BDCM.

Finally, in contrast to the F344 strain, this research group demonstrated that Long Evans rats, and especially Sprague-Dawley rats, are remarkably less sensitive to BDCM-induced pregnancy loss. It is planned to pursue these differences in strain sensitivity as a research tool that may provide insights into susceptible subpopulations as well as BDCM's mode of action.

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<sup>1</sup>Waller K, Swan SH, DeLorenze G, Hopkins B. Trihalomethanes in drinking water and spontaneous abortion. *Epidemiology* 1998;9:134-140.

## Assessing Consequences of Embryonic Exposure to Methoxychlor: Neuroendocrine and Behavioral Measures

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Endocrine-disrupting chemicals (EDCs) include pesticides, herbicides, and other chemicals that interact with endocrine systems. This research project focused on methoxychlor (MXC) and other EDCs, as well as on effects of the estrogenic compounds and on estradiol as a positive control to assess potential impact of estrogenic EDCs. The male Japanese quail is exquisitely sensitive to the effects of exogenous estradiol during sexual differentiation, resulting in impaired sexual behavior and reduced fertility. Moreover, exposure of two successive generations affected the untreated offspring.

A series of studies were conducted on Japanese quail, with exposure to MXC, a widely used pesticide. Exposure was either by egg injection (0, 150, or 300 µg) at embryonic day 4 or via the diet (0, 0.5, or 5 ppm) in a two-generation experimental design. Embryonic exposure resulted in impaired sexual behavior in the adult male Japanese quail. Moreover, long-term effects on hypothalamic catecholamine systems and on the gonadotropin-releasing hormone-I system were detected. Dietary exposure did not affect the parent generation; however, both the F1 (MXC treated) and F2 (no MXC treatment) offspring showed effects of MXC exposure. Although fertility and other general indicators of reproduction did not appear to be affected, other endpoints critical to reproduction were impacted by MXC, including plasma steroid hormones, sexual behavior, and sexual maturation. These data suggest that the embryo is the most sensitive stage for exposure to EDCs, with effects on a subset of reproductive endpoints.

Bobwhite quail have been a species of choice for assessing effects of toxic compounds in birds. However, little is known about EDC effects on reproductive endpoints in Bobwhite quail. Therefore, Bobwhite and Japanese quail were simultaneously compared in a two-generation study, with dietary exposure to MXC (0, 5 ppm, and 10 ppm). The parent generation was raised under short photoperiod, paired, and transferred to long days (16L:8D) with initiation of treatment. Basic measures of health and reproduction were monitored, including feed intake, egg production, fertility, and offspring viability.

Chicks (F1) were raised on the same diet as their parents and observed for sexual maturation, reproductive behavior, and endocrine endpoints. Results showed species differences in maturation, with Bobwhite quail requiring 3-4 weeks longer to achieve sexual maturity. Neither species showed effects of 5 ppm MXC on egg production, fertility, body weight, feed intake, or chick viability. However, Japanese quail fed MXC matured more slowly, suggesting that the treatment interfered with activation of reproduction. A separation test was used in young chicks to assess motivation to rejoin siblings. These data demonstrate the importance of examining individual responses to EDCs. In summary, neuroendocrine and behavioral measures are likely to provide reliable endpoints that relate to embryonic EDC exposure. These measures are particularly relevant for consideration in assessing the long-term impact of EDCs on birds because these endpoints are sexually dimorphic and organized under the influence of steroid hormones during embryonic development.



## **Endocrine Disruptors and Testis Development**

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Endocrine disruptors have been shown to influence male reproduction by causing abnormal sperm numbers and fertility. One of the most sensitive periods to endocrine disruptor exposure is during embryonic development. The objective of the current research is to investigate the mechanism of action of a model endocrine disruptor on male reproduction with a focus on testis development. A rodent model system is used to provide insight into the mechanistic aspects of endocrine disruptor action. The model endocrine disruptor tested is methoxychlor, which has metabolites that are both weak estrogenic and anti-androgenic compounds. Therefore, this model endocrine disruptor allows consideration of both estrogenic and anti-androgenic endocrine disruptor actions. Comparisons with standard estrogenic and androgenic steroid agonists and antagonists also are made. The objective is to obtain insight into the molecular, cellular, and physiological actions of endocrine disruptors on male reproduction. The hypothesis tested is that endocrine disruptors (i.e., methoxychlor) influence embryonic testis development via steroid receptors to interfere with critical growth factor-mediated cell-cell interactions that result in abnormal germ cell differentiation through epigenetic effects (e.g., DNA methylation), and that this subsequently influences adult spermatogenesis and is transgenerational through the germline.

Studies have shown that methoxychlor can affect embryonic testis development at the time of testis morphogenesis, and that this causes an increase in germ cell apoptosis in the adult. Interestingly, observations suggest this abnormal spermatogenesis is transgenerational, and preliminary data suggest altered DNA methylation of the germline may be the epigenetic action of the endocrine

disruptor. Preliminary studies also have demonstrated that two families of paracrine growth factors directly influence testis development at the time of methoxychlor action. Abnormal testis development and germ cell differentiation caused by endocrine disruptors may be due in part to inappropriate control of these critical growth factor-mediated cell-cell interactions. Preliminary studies indicate that the transforming growth factor (TGF) families are critical for embryonic testis growth. Inhibition of the TGF factors blocks testis growth and results in abnormal testis development and potential subfertility. Preliminary studies also indicate that the neurotrophin (NT) family of factors (i.e., NT3) has a critical role in the morphogenesis of testis development (i.e., sex cord or seminiferous tubule formation). This non-neuronal action of the NTs when blocked inhibited normal testis development and morphogenesis, which may result in abnormal germ cell differentiation and subfertility in the adult male. Methoxychlor was found to alter the expression of these growth factors. Abnormal control of critical testis cell-cell interactions after treatment with different doses of endocrine disruptors is anticipated to influence germ cell development and male fertility.

Information obtained provides insight into how environmental toxins (i.e., methoxychlor) may impair male fertility by adversely effecting gonadal NTs and transforming growth factors. These studies develop a better understanding of the mechanistic aspects of how endocrine disruptors influence reproductive function (i.e., testis growth and function). The research will be discussed to extrapolate and provide insight into the impact of endocrine disruptors on human development, reproduction, and health.

## Effects of Early Developmental Exposure to Endocrine-Disrupting Chemicals on Reproductive Function in the Adult Male: Rabbit Model

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This research project focused on developmental and long-term reproductive sequelae of prenatal plus infantile exposure of male rabbits to pesticides that are known to competitively bind to androgen receptor and thus exert anti-androgenic activity. Rabbits were chosen as a model because this species, unlike rodents, has a relatively long quiescent period of reproductive development before puberty, mimicking that of humans. Moreover, longitudinal evaluations of endocrine profiles, sexual capacity, and semen quality are feasible in rabbits.

Groups (n = 7-10) of Dutch-belted rabbits were treated daily beginning from gestation day 15 through post-kidling week 4 with p,p'-dichlorodiphenyltrichloroethane (p,p'-DDT), vinclozolin, or a mixture of both. The chemicals were administered orally in corn syrup at 0 (control), 25 (low dose), or 250 (high dose)  $\mu\text{mol/kg}$  body weight. Testicular descent to a scrotal-dependent position was monitored by palpation, beginning at 4 weeks after birth. One of the 15 low-DDT pups, 4 of the 16 high-DDT pups, 1 of the 17 low-mixture pups, and 3 of the 18 high-mixture pups were cryptorchid. Histological evaluation revealed the presence of atypical germ cells, some resembling carcinoma *in situ*, in the undescended testes. Beginning at 20 weeks of age, six seminal ejaculates were collected, one every 3-4 days, by using an artificial vagina. Ability to accomplish ejaculation was recorded by monitoring outcome (interest to mount, penile erection, and time taken to ejaculate) within 3 minutes after introduction of a female teaser. Two of the 7 low-vinclozolin groups and 2 of the 9 low-mixture groups showed absolutely no

sexual interest and never ejaculated. One each of 8 low-DDT and 8 low-vinclozolin rabbits and 3 out of 10 high-vinclozolin rabbits failed to ejaculate at least once. The serum concentrations of luteinizing hormone and testosterone in any of the treatment groups did not differ from that of the control ( $p > 0.01$ ). However, follicle-stimulating hormone was consistently lower in vinclozolin-treated rabbits ( $p < 0.05$ ). This indicates that the impairment of androgen-dependent events resulted not from lack of testosterone, but possibly from unavailability of the receptor because of either its downregulation or chronic occupancy by the pesticides or their metabolites.

In summary:

- p,p'-DDT, but not vinclozolin, caused cryptorchidism. Atypical germ cells that resemble carcinoma *in situ* were evident in DDT-induced undescended testes.
- Developmental exposure to anti-androgenic pesticides caused sexual dysfunction in the male; lack of sexual interest and ejaculation failures were more prevalent in vinclozolin-exposed rabbits.
- Impairment of androgen-dependent events may have resulted from unavailability or dysfunction of androgen receptor because serum testosterone was unaffected.

These results indicate that exposure of males to anti-androgenic pesticides during critical periods of development impairs reproductive function as adults.

**Exposure, Risk Assessment, and  
Risk Management**

## Cross Species Mode of Action Information Assessment for Bisphenol A

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The goal of this project was to identify mode of action (MOA) information for Bisphenol A (BPA) across animal species and to explore how to integrate this information for the evaluation of ecological and human health risks. This approach is predicated on the idea that common MOAs are a function of evolutionary relationships. BPA was selected because it is a high production volume chemical that can act as an endocrine-disrupting chemical (EDC) and there are some effects data in some nonmammalian and invertebrate species. BPA MOA information for developmental and reproductive effects was gathered from the published literature. BPA *in vivo* effects data were identified for only 17 species (of the total 9 or 10 million) representing 7 animal classes: gastropods, crustaceans, insects, amphibians, fish, birds, and mammals. For invertebrates, the MOA for BPA could not be determined. In gastropods, BPA treatment led to sexual differentiation effects, but data to establish an MOA were lacking. For the crustaceans, no consistent effects were observed in daphnids; in copepods, effects were consistent with an estrogen (E) agonist MOA; and for insects, data were dissimilar from E2 effects and data from an *in vitro* assay suggest an ecdysone antagonist MOA.

Although some invertebrate species showed effects from E or BPA exposure, the significance of the findings is unclear because the role of E (or E analogs) in normal invertebrate development is not known. Within the vertebrates, *in vitro* studies found that BPA competitively binds to the estrogen receptor (ER) of reptiles, amphibians, fish, birds, and mammals at a much lower affinity (250-12,500 times lower) than ethinyl estradiol. For amphibians, some developmental effects data were consistent with BPA acting as an E agonist, and *in vitro* data suggest a thyroid

hormone bioavailability MOA. In birds, *in ovo* BPA treatment led to some significant sexual differentiation effects, consistent with an E agonist MOA. In fish, BPA *in vivo* study findings were consistent with an E agonist, androgen (A) antagonist, an A agonist, steroid hormone bioavailability, and/or a nonendocrine MOA. Mechanistic data in fish support BPA treatment leading to an increased E activity, including E agonism. Mammalian *in vivo* effects data, limited to rat and mice, are consistent with an E agonist, A antagonist, A agonist, altered E bioavailability, altered A bioavailability, altered thyroid hormone, and/or altered prolactin hormone MOA; whereas most mechanistic studies support an E agonist and one study supports a decreased A bioavailability MOA. Human and rodent *in vitro* ER-binding data indicate that BPA can act as an E agonist.

Together, the data indicate that BPA exposure can elicit effects in vertebrate and invertebrate species at sublethal concentrations, and the majority of the evidence supports BPA acting to increase E or E analog activity with mechanistic studies supporting E agonism. Limitations of the BPA assessment were the small number of species with data, the focus of most studies on the E MOA, and the lack of knowledge of the role of hormones in invertebrate sexual differentiation as well as the role of estrogens in mammalian male development. Studies to investigate whether BPA affects alternative or multiple MOAs at different doses and under different conditions are needed. When BPA MOA and effects data for additional animal species have been generated, the MOA evolutionary relationships among species could be used to make predictions of MOA to untested species.

## **Overview of Intramural Exposure Research Program**

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The National Exposure Research Laboratory (NERL) conducts research to develop and validate exposure tools for characterizing human and ecological exposures to endocrine-disrupting chemicals (EDCs). NERL's Methods Development Research Program includes enhanced analytical methods for assessing ecological exposures to alkylphenols and related compounds as well as innovative DNA microarray techniques to rapidly identify first-order ecological effects resulting from exposures to estrogenic compounds. Large-scale exposure field measurement studies also are being planned throughout the United States. Several studies have been conducted to assess children's exposures to EDCs, pesticides, and other organic pollutants. A longitudinal study examining children's aggregate

exposures to EDCs and other pollutants is being planned for Jacksonville, FL, in Fiscal Years 2003-2005. Ecological field studies also are being conducted to characterize the extent of EDCs and pesticide exposures in eastern, midwestern, and western ecological watersheds. Exposure modeling research includes enhancements of the current AGDISP (spray drift) model to include additional application techniques, secondary volatilization, and regional transport of agricultural pesticides. NERL's primary ecological and human exposure models (PRZM/EXAMS and SHEDS/ERDEM, respectively) also are being upgraded for use in characterizing and assessing EDC exposures. This overview presentation will highlight NERL's current and future EDC exposure research programs.

## Development of Molecular Diagnostic Indicators of Exposure to Estrogenic and Androgenic Endocrine-Disrupting Chemicals

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The extent to which humans and wildlife are exposed to endocrine-disrupting chemicals (EDCs) is an important focus of environmental research. This work has been directed toward the development of molecular indicators diagnostic for exposure to EDCs in freshwater fish. Research includes the discovery of genes indicative of environmental exposure in the U.S. Environmental Protection Agency's (EPA) long-established aquatic toxicological organism, the fathead minnow (*Pimephales promelas*). Novel cDNAs and gene sequences will be used in DNA microarray analyses for pattern identification of stressor-specific, differentially up- and downregulated genes. The methods currently used to discover genes in this organism, for which few annotated nucleic acid sequences exist, are cDNA subtraction libraries, differential display, exploiting polymerase chain reaction (PCR) primers for known genes of other members of the family *Cyprinidae*, and use of degenerate PCR primers designed from regions of moderate protein homology. To date, hundreds of fathead minnow cDNA sequences, resulting from exposure to estrogenic compounds, have been isolated by subtractive hybridization cloning, and a preliminary glass-based DNA microarray has been constructed to which gene sequences will be added as further discovery and characterization proceed.

As the number of genes that will detect stressor-specific transcriptome changes continues to expand, future microarray spot printing of covalently bound DNA will use overlapping oligonucleotides as hybridization targets, replacing the current cDNA platform. This approach will increase efficiency of hybridization, sensitivity of detection, and aid

in addressing inherent issues of reproducibility. Single or multiple genes noted as being differentially expressed in microarray analyses then will be used in separate studies to measure bioavailable stressors in the laboratory and field. These analyses will be accomplished by "real time," quantitative PCR. The expression of some genes (e.g., vitellogenin in male fish and the androgen receptor gene) that indicate exposure to estrogens and androgens, respectively, have been experimentally confirmed in the laboratory and used in a number of environmental EDC evaluations.

Currently, field and laboratory studies are being conducted, including, but not limited to: (1) mesocosm exposure experiments to measure variation in estrogen bioavailability as influenced by primary productivity in aquatic ecosystems, (2) monitoring studies of potential estrogenic EDCs in effluent samples taken from 50 U.S. sewage treatment plants, (3) exposure and effects studies of androgenic activity in effluents collected at concentrated animal feeding operations, and (4) surface water and effluent monitoring studies in EPA Region 9, subsequent to a recently initiated cooperative technology transfer program between the National Exposure Research Laboratory/Office of Research and Development and EPA Region 9. Positive results in these studies present a range of possibilities for those genes identified by DNA microarray analyses to be critical components of toxicity pathways for stressors having various modes of action. The ability to discriminate bioavailability of mixture components and their association with adverse effects, downstream from these early molecular events, presents new ground to be broken in EDC risk assessment.

## Intramural Research on Risk Management of Wastewater Treatment Sources

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Extensive data in the literature indicate that certain endocrine-disrupting chemicals (EDCs) commonly appear in surface waters. For example, a recently published report from the U.S. Geological Survey's National Reconnaissance Study showed that 90 percent and 70 percent of the surface water locations tested in the United States showed measurable levels of steroid hormones or detergent metabolites, respectively. Other published data sets have revealed that municipal wastewater treatment plants receive steroid hormones and alkylphenol ethoxylate surfactants, and that in the plants, the hormones often are not completely destroyed and the surfactants are biotrans-formed into estrogenic metabolites. Wastewater treatment plants are potential sources of EDCs to the environment by two routes: aqueous effluent and the disposal of biosolids (sludge). A major portion of the U.S. Environmental Protection Agency's research program on risk management of EDCs is focused on understanding wastewater treatment plants as sources of EDCs to the environment and, as needed, developing approaches to minimize this source. To address this complex problem, several projects have been undertaken:

- **Fate of EDCs in Wastewater Treatment Processes.** A study is underway to understand the fate of EDCs in the various unit processes within treatment plants by constructing and operating two secondary treatment pilot plants (one with aerobic digestion and the other with anaerobic digestion). To date, the plants are fully operational, and analytical methods have been developed for all sampling streams. Steroid hormones and alkylphenol ethoxylate surfactants are to be introduced to the plant this month.
- **Removal of EDCs by Sludge Digesters.** As part of a larger National Risk Management Research Labora-

tory (NRMRL) biosolids research study, this research project will sample several full-scale sludge digesters to correlate the ability of digesters to remove steroid hormones and alkylphenols in sludge as a function of operational process variables.

- **Removal of EDCs by Land Application of Biosolids.** Most commonly, biosolids are disposed of by land application. Also part of the NRMRL biosolids study, the ability of land application to treat steroid hormones and alkylphenols in biosolids will be evaluated for typical land application techniques.
- **Characterizing Wastewater Treatment Plants and Their Effluents.** A national screening study is underway by NRMRL, National Exposure Research Laboratory, and the Regional offices, in which up to 50 municipal treatment plant effluents will be screened for endocrine-disrupting character and related to characteristics of the plant. The endocrine-disrupting character will be determined by a vitellogenin gene expression assay and by analytical chemistry for steroid hormones and alkylphenols. To date, 30 effluents have been assessed.
- **Potential of Sediments To Remove EDCs.** EDCs that are not removed by treatment may partition from the receiving surface water in aquatic sediments. Laboratory studies are underway to evaluate the ability of sediments to transform or destroy steroid hormones and alkylphenols under various typical redox conditions.

The presentation will summarize the wastewater treatment program including its goals, approaches, and results to date.

## Endocrine-Disrupting Chemicals Related to Feminization of Males

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Observations in wildlife on the potential of certain environmental chemicals to modulate endocrine-regulated processes have led researchers to question whether similar abnormalities are occurring in humans. Currently, there is a growing realization that a wide range of anti-androgenic endocrine-disrupting chemicals (EDCs) is associated with feminization of birds, fish, and alligators. Male fish with DNA code of the Y chromosome and elevated vitellogenin levels have decreased serum testosterone concentrations. Feminization of veterinary animals is not uncommon. Various degrees of male genital abnormalities from mild to moderate hypospadias, unilateral or bilateral cryptorchidism, and poorly developed testes are common in dogs, goats, horses, and other domestic animals.

Parental *in utero* or perinatal exposure of experimental animals to EDCs, namely dichlorodiphenyltrichloroethane, dichlorodiphenyldichloroethylene, di(2-ethylhexyl) phthalate, flutamide, and vinclozolin, can result in hypospadias, cryptorchidism, and other male sexual abnormalities. Epidemiologic studies have reported an increased risk of male genital malformation in children of workers exposed occupationally to pesticides and clustering of cryptorchidism in areas of intensive agriculture/horticulture where various types of

pesticides are used. A marked increase in the incidence of hypospadias in the United States, European countries, and Japan was observed during the 1960s, 1970s, and 1980s at a time when exposure to anti-androgenic EDC levels in the environment was high. Hypospadias and cryptorchidism are occurring approximately at a rate of 1 in 125 male births in the United States. In humans, the male sexual disorders, including hypospadias and cryptorchidism, are symptoms of one underlying entity, the testicular feminization syndrome. Animal data indicate that the parental or *in utero* or perinatal exposure to EDCs can result in various degrees of feminization of male offsprings with testicular feminization expressed as hypospadias, cryptorchidism with undescended testis, and related intersex conditions.

To examine whether male sexual abnormalities and their different degrees of manifestations are associated with parental, *in utero* (at different stages of organogenesis), or perinatal exposures to anti-androgenic EDCs, the U.S. Environmental Protection Agency will convene a workshop in Cincinnati, OH, on December 4 and 5, 2002. The primary objective of this workshop is to determine the rationale and approach for exposure and risk assessments of environmental anti-androgenic EDCs.



## Overview of the Intramural Risk Management Research Program

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This presentation will provide a summary of the risk management portion of the Office of Research and Development's endocrine-disrupting chemicals (EDCs) research program, including its motivation, goals, planning efforts, and resulting research areas.

In an emerging research area such as EDCs, risk management (RM) research and risk assessment (effects, exposure, characterization) research can and should be conducted in parallel. Early or tentative effects and exposure information can point to the identification and development of risk management approaches that may prevent or control risks with high effectiveness at low cost. These early risk management approaches may possess relatively large uncertainty in their effectiveness at reducing risk because the risk information is relatively uncertain. However, if risk managers must make a decision based only on limited risk information, risk management approaches with large uncertainties may be more attractive than no options. As the knowledge of risk matures, the approaches for risk management become increasingly certain.

Consistent with this philosophy, the RM research program began with the development of the *Risk Management Evaluation (RME) of Endocrine Disrupting Chemicals*. This RME provides a snapshot in time of the current state of risk management (i.e., what currently available risk management approaches can be adapted to address EDCs, and what new RM technologies or strategies need development?). This document should be available to the public by the end of 2002. Because risk information is continually evolving and risk management technologies and approaches are continually growing and

maturing, the RME for EDCs will be updated on a regular basis.

The RME provided a list of research questions that have been used to plan the RM research program. The program is characterizing sources of EDCs to the environment, developing strategies to minimize these sources, and investigating strategies to remediate EDCs in environmental reservoirs. Specifically, research is being conducted in the following areas:

- Studying wastewater (sewage) treatment as a source of EDCs, and developing approaches to improve treatment as necessary.
- Characterizing concentrated animal feeding operation as sources of EDCs and developing animal waste treatment approaches as necessary.
- Characterizing combustion processes as sources of EDCs, and developing improvements to the process to minimize this source.
- Studying the natural attenuation of alkylphenols in sediment impacted by wastewater treatment plant outfalls.
- Evaluating the ability of conventional and advanced drinking water treatment plants processes to remove EDCs, and improving these processes if necessary.
- Developing pollution prevention tools that can be used to evaluate and nominate substitute chemicals for EDCs currently in use.
- Adaptation of EDC screening bioassays for use in determining the performance of RM technologies.

## **Field Studies**

## Frog Deformities: Role of Endocrine Disruptors During Development

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There is considerable evidence that wild animals have suffered adverse consequences from exposure to environmental chemicals that interact with components of the endocrine system. The high incidence of deformed frogs in North America, coupled with the worldwide decline in the occurrence of amphibian species, suggests that environmental modification is negatively impacting amphibian populations. Although the cause of amphibian declines in relatively pristine environments remains unknown, there is an emerging consensus that the increasing prevalence of deformed frogs is the result of a waterborne contaminant that has appeared, or reached a critical concentration, in recent years. The objective of this research project is to assess the significance of endocrine disruptors that activate retinoid-signaling pathways for their role in causing limb developmental deformities in frogs, and to understand their mechanism of action to assess their implications for human health.

This research group analyzed the skeletal dysplasias observed in severely affected frogs from Minnesota and identified two classes of common limb abnormalities. First, supernumerary or absent limbs, suggesting that the process of limb initiation is being affected. Second, skeletal abnormalities, including truncated and phocomelic limbs, suggesting that limb growth and pattern formation also are being modified. In the phocomelic limbs, the skeletal elements are folded back on themselves, such that the proximal and distal ends of the bone lie adjacent to one another and the mid-portion of the bone projects laterally, forming a “bony triangle.” Retinoid treatment at sensitive stages phenocopies deformities in wild frogs. The effects of treating a range of stages of larval development with retinoids was tested, and it was found that all the deformities that are observed in wild populations of frogs can be induced by experimental exposure to retinoids. Using the parameters established in these studies, a developmental toxicology assay has been developed to screen the activity of a number of chemicals

known to be present at sites where deformed frogs are found.

If environmental retinoids are the cause of frog deformities, then retinoids will be found at sites where deformed frogs are found. To test this hypothesis, hydrophobic substances will be extracted from water samples, and then fractionated by high-performance liquid chromatography and tested for their ability to activate the retinoic acid receptor in transient transfection assays. Active fractions will be purified to homogeneity as judged by ultraviolet absorption spectra and then analyzed by electrospray and electron impact mass spectroscopy for exact mass determination. Candidate compounds thus identified then will be retested in the reporter and animal assay to verify biological activity. Similar activity peaks have been discovered in water samples from a vernal pond in Mission Viejo, California, as in the permanent lake being studied in Minnesota.

In addition to the laboratory studies, these researchers have continued to monitor field sites in Minnesota. At the Crow Wing County, Minnesota, site, all ranid frog species have declined during the 3 years of the study. This past spring, no leopard frog or green frog calls were heard, and only scattered mink frog calls were heard. Only one leopard frog juvenile was found at the site during the entire 2000 season (compared to 562 in 1997, a year of similar sampling effort, for example). Mink frog capture success also was decreased; only 74 juveniles were captured in 2000, compared to 365 in 1997. The mink frog was the only species with enough juvenile captures to calculate meaningful malformation frequencies. The total malformation frequency among juveniles in 2000 was 18 percent, apparently lower than 1996-1999, when the frequency ranged from 50-75 percent, but still much higher than the working “background” frequency of 1 percent.

## The Mechanisms and Effects of Endocrine Disruption on Infertility in the Bonnethead Shark on Florida's Gulf Coast

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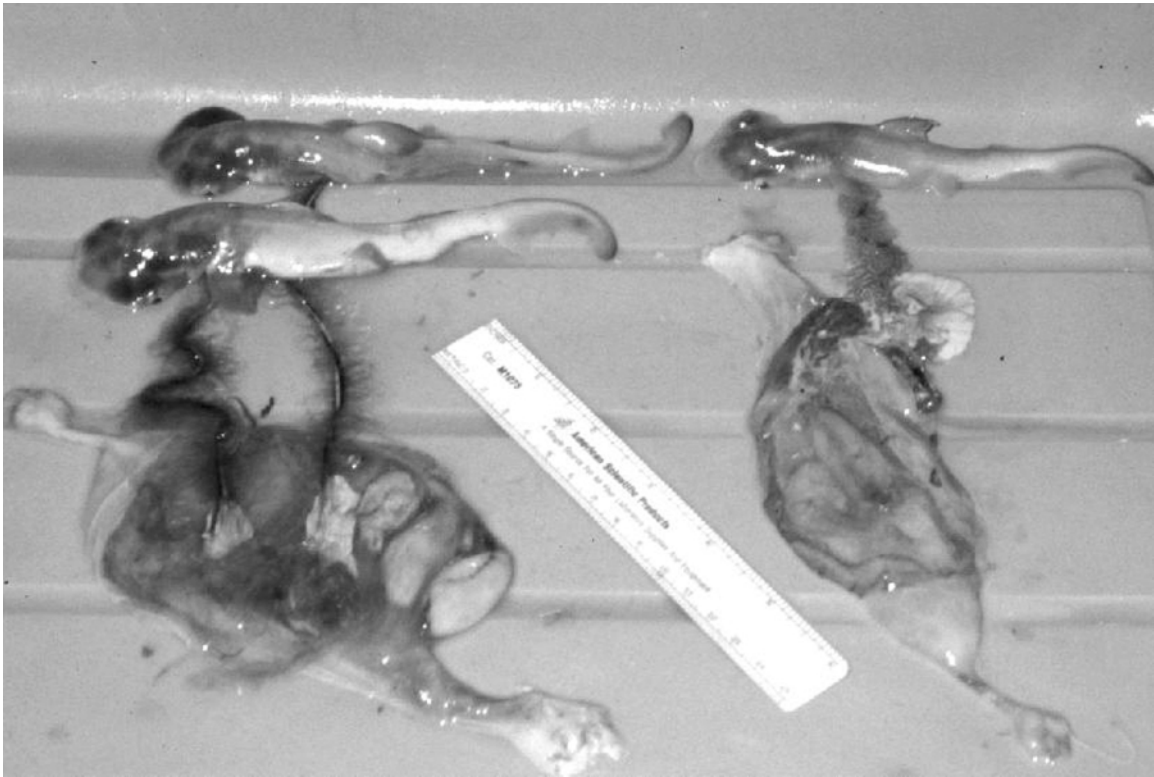
Previous studies have demonstrated a high frequency of infertile ova in the uteri of pregnant bonnethead sharks (see Figure 1) from the Tampa Bay area, a highly industrialized region on Florida's Gulf Coast. Whereas infertility typically is rare in most shark species, its occurrence in approximately 75 percent of pregnant bonnethead sharks examined in recent surveys of affected populations suggests that it may have detrimental effects on population growth. The objectives of this study were to: (1) determine if infertility observed in these populations is associated with disruption of the endocrine system; (2) identify the mechanism(s) underlying the production of infertile ova; (3) determine if infertility is correlated with levels of environmental contaminants in shark tissues; and (4) estimate the effects that infertility may have on the rate of population growth.

To address these objectives, levels of environmental contaminants, trends in growth and reproduction, and rates of increase for three separate populations of bonnethead sharks on Florida's Gulf Coast were investigated. Sharks were collected from three dissimilar geographical regions: (1) the Anclote River, a site adjacent to Tampa Bay that is known to contain high levels of environmental pollutants and high rates of infertility in resident shark populations; (2) Apalachicola Bay, a site on the northwest coast of Florida that possesses moderate levels of environmental contamination; and (3) Florida Bay, a site that is known to possess relatively low levels of environmental pollutants and low rates of shark infertility.

The reproductive competence of mature male and female sharks was evaluated using a variety of indices, in-

cluding measures of gonadal development, semen quality, female sperm storage, and fertility. The relationship between reproductive success and endocrine function was investigated using measurements of serum steroid concentrations. Associations between reproductive success and levels of environmental contamination were evaluated by measuring the concentrations of organochlorine pesticides (OCs) and polychlorinated biphenyls (PCBs) in shark tissues. Lastly, differences in population growth were determined by incorporating estimates of natural mortality, age at maturity, lifespan, and fecundity (which is influenced by fertility rate) into demographic models for the three study populations.

The reproductive biology of male bonnethead sharks from the three study sites did not differ significantly. In contrast, the ability of female sharks to store spermatozoa prior to fertilization was significantly lower in sharks from the Anclote River. Differences in sperm storage, fertility and, perhaps, other undetected disparities in female reproduction appear to be related to differences in endocrine function, based on low serum concentrations of 17 $\beta$ -estradiol measured in Anclote River sharks specifically during the period of sperm storage and vitellogenesis. An association between high rates of infertility and levels of contaminant exposure was observed, based on significantly elevated concentrations of total PCBs and more than 10 OCs in Anclote River sharks. Differences in fertility do not appear to have a profound effect on population growth, based on demographic analyses. However, the effects of infertility on population growth are likely to be greater in most other regional shark species, which display lower rates of population increase.



**Figure 1.** Reproductive tract and embryos from a pregnant female bonnethead shark from the Tampa Bay region of Florida's Gulf Coast. Several infertile eggs also are present in both uteri. Embryos are attached to uteri by placental-like attachments, which provide nourishment during the second half of pregnancy (embryos are dependent on yolk for nourishment prior to development of these connections).

## Endocrine Disruption in Marine Gastropods by Environmental Chemical Mixtures

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The masculinization of female snails, termed imposex, occurs worldwide in more than 150 species following exposure to low levels of tributyltin (TBT) (as low as 1 ng/L).<sup>1</sup> Females exposed to TBT grow accessory sex organs (ASO), including sperm ducts, seminal vesicles, external sperm grooves, and most notably, penises. At least two mechanisms have been proposed for the development of imposex: one involves the abnormal release of neurohormones that control sexual maturation and reproduction in molluscs; the other is based on the vertebrate model of steroid hormone regulation.

The first model of imposex induction centers on the concept that TBT acts on the ganglia of snails to alter the expression of neuropeptide hormones. TBT is thought to control the expression/activity of penis morphogenic factor (PMF), a neuropeptide that controls the production of male accessory sex organs in gastropods. Injection of a putative PMF into female mud snails resulted in a significant induction of imposex. In addition, snails exposed to TBT contain higher levels of immunodelectable APGWamide (the putative PMF). This laboratory also has shown that TBT decreases the number of egg capsules laid by female snails. Egg laying is controlled by a 36-amino acid neuropeptide (ELH) produced in the gan-

glia of gastropods. It is possible that TBT functions on a signal transduction pathway common to both peptide hormones.

In the second model of imposex induction, TBT is proposed to function as a noncompetitive inhibitor of cytochrome P450 metabolism. This enzymatic inhibition blocks the metabolism/conversion of steroids, ultimately leading to a buildup of testosterone and the subsequent development of ASO in female snails.<sup>2</sup> Studies in this laboratory have shown that TBT significantly reduces the levels of each steroid (pregnenolone, progesterone, and androstenedione) produced in the mud snail. In addition, there is an approximately 50 percent reduction in aromatase activity in snails after exposure to 20 ng/L of TBT for 45 days and in environmentally exposed animals.

From these studies, it is evident that both mechanisms play a role in the induction of imposex in gastropods, even at extremely low doses of TBT. It is likely that a positive feedback loop mechanism stimulated by both processes functions in the development and maintenance of these structures. TBT would stimulate the release of PMF to induce imposex in females, while steroids would act on the feedback loop to maintain these structures.

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<sup>1</sup>Oberdörster E, McClellan-Green P. The neuropeptide APGWamide induces imposex in the mud snail, *Ilyanassa obsoleta*. *Peptides* 2000;21(9):1323-30.

<sup>2</sup>Fent K. Ecotoxicology of organotin compounds. *Crit Rev Toxicol* 1996;26(1):1-117.

## **Effects of PCBs on Herring Gulls in the Field and Chickens in the Laboratory**

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The objectives of this study were to investigate the effects of polychlorinated biphenyl (PCB) exposure on thyroid function in developing Herring gulls from Great Lakes (GL) sites and in developing chickens from PCB-dosed eggs. Herring gulls, a fish-eating top-predator, have been used as a sentinel species to monitor contaminant effects in the GL for more than 30 years. Because GL gulls have had thyroid hypertrophy and histopathology, and because PCBs disrupt thyroid function in laboratory mammals, it has been speculated that PCB-exposed GL gulls may have depressed thyroid function despite decreases in PCBs and other organochlorines since the 1970s.

This research group assessed organismal thyroid status based on plasma thyroid hormones (THs), thyroid gland (TG) function based on TG-TH content, and activation of the hypothalamic-pituitary-thyroid (HPT) axis based on TG weight (TG hypertrophy indicates a negative feedback response to low circulating THs). Some of these studies have addressed mechanisms by which thyroid disruption may occur (enhanced liver glucuronidation of T<sub>4</sub>, and displacement of THs from plasma-binding proteins) and whether hormone activation responses may be compensating for decreases in THs (brain 5' deiodinase activity).

Herring gulls collected from GL in 1998-2000 show that both pipping embryos and prefledglings from high PCB sites have severe depletion of TG-TH stores compared to gulls at the reference site. However, in some cases birds from high PCB sites were able to maintain thyroid status comparable to that at the reference site. Embryos and prefledglings differed in their HPT axis re-

sponse to PCB exposure. Adults, sampled at two high PCB sites and a low PCB site in the GL and the reference colony in 2001, did not differ in organismal thyroid status across sites, but those from the high PCB sites had hypertrophied TGs and TG-TH depletion. The depletion of TG-THs in PCB-exposed birds suggests that they have greatly diminished capacity for thyroid responses in relation to environmental change. Thus, environmental stressors may have different effects on gull populations at high PCB sites, compared with reference sites.

Studies of chicken embryos and chicks hatched from eggs dosed with PCB 126, PCB 77, or Aroclor 1254 suggest that embryonic exposure to these PCB treatments has little or no effect on thyroid function. The PCB 126 and 77 doses used caused immune system effects, and the highest dose caused considerable mortality (evaluations from K. Grasman's laboratory).

Studies of mechanisms of thyroid disruption focused on PCB effects on the Phase II liver biotransformation enzyme uridine diphosphate glucuronosyltransferase (UDP-GT) and on the displacement of THs from binding proteins. No evidence of hepatic UDP-GT induction by PCB 126 or 77 in chicken embryos or chicks hatched from PCB-dosed eggs was found. Because UDP-GT induction resulted in enhanced T<sub>4</sub> metabolism and excretion is a key mechanism by which PCBs alter thyroid function in mammals, these results suggest that PCBs do not have equivalent effects on birds and mammals. Preliminary studies of GL gull plasma suggest that PCBs may be displacing THs from their binding proteins at high PCB sites.

## Effects of Exposure to Environmental Estrogens on Reproductive Parameters in a Marine Fish, *Tautogolabrus adspersus*

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Estradiol ( $E_2$ ), ethynylestradiol ( $EE_2$ ), and estrone ( $E_4$ ) are steroidal estrogens that are released into the aquatic environment in sewage treatment effluent. To determine whether these estrogens could impact reproductive parameters in a model fish species, actively spawning male and female cunner (*Tautogolabrus adspersus*) were exposed in the laboratory by implanting  $E_2$ ,  $EE_2$ , or  $E_4$  subcutaneously in a slow-release matrix. A separate experiment was conducted with each of the three estrogens. Experiments consisted of four treatments: control (implant matrix only) and three concentrations of estrogen (0.05, 0.5, or 2.5 mg/kg). Four replicate tanks, each with three females and one or two males, were used per treatment. Reproductive success of fish before and after implantation was assessed through daily measurements of egg production, number of fertile eggs, and number of viable developing eggs. At the end of a 2-week exposure period, blood was drawn from each fish for plasma steroid hormone and vitellogenin analysis. Fish then were dissected, gonads weighed, and select tissues preserved for later histopathological analysis.

Egg production prior to the implantation procedure was not significantly different among the three experiments, averaging about 280 eggs/gram female/day. Egg viability prior to implantation ranged from 17 to 20 percent, while mean egg fertility ranged from 25 to 52 percent. After implantation, only egg production in the 2.5 mg/kg  $EE_2$  treatment was significantly lower than in the controls. Notable, but not statistically significant, decreases in egg production relative to controls were observed in the 2.5 mg/kg  $E_2$  treatment, and in the 0.5 and 2.5 mg/kg  $E_4$  treatments. Neither mean percent viability

nor percent fertility was significantly different than controls in any estrogen treatment. All estrogen treatments induced production of the female protein vitellogenin (Vtg) in males, but  $EE_2$  more so than the others. In males from the highest concentration of  $EE_2$ , average plasma concentration of Vtg (480 mg/mL) was approximately 40 times (12 mg/mL) that in the high  $E_2$  treatment and 9 times (53 mg/mL) that in the high  $E_4$  treatment. Overall, these results indicate that short-term exposure of mature cunner to estrogens induces Vtg production in males and may decrease egg production in females.

To investigate whether the presence of male Vtg is a reliable indicator of decreased reproductive success in mature fish, data on egg production, egg viability, egg fertility, sperm motility, and male Vtg concentrations from the 2-week exposure experiments were combined with results of earlier 8-week exposure experiments. All males, including two with Vtg levels exceeding 300 mg/mL, produced motile sperm. Neither percent fertile eggs nor percent viable eggs produced by reproductively active fish demonstrated a significant correlation with male Vtg concentrations. Male gonadosomatic index and average daily egg production by females showed significant, but weak, negative linear correlation with male Vtg concentrations. Results suggest that male Vtg expression is not a reliable indicator of male reproductive dysfunction in adult cunner exposed to estrogens during their reproductive season, at least in relation to their capacity to produce motile sperm or to fertilize eggs. In some cases, male Vtg expression may serve as an indicator of reduced reproductive function in females exposed to estrogens at the same time.



## **Mechanisms of Action**

## Polymerase Chain Reaction-Differential Display (PCR-DD) Identifies a Subset of Stage-Dependent and Toxicant-Regulated Genes During Spermatogenesis: The Shark Testis Model

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Due to a cystic mode of spermatogenesis and a simple linear arrangement of developing spermatocysts across the diameter of the testis, the spiny dogfish shark (*Squalus acanthias*) is an advantageous model for stage-by-stage analysis of factors and mechanisms controlling spermatogenesis. In previous studies, this research group reported that estrogen receptor (ER) and androgen receptor (AR) binding activities are concentrated in the germinal zone (GZ) and premeiotic (PrM) regions, where spermatocysts (follicle-like germ cell/Sertoli cell units) are in the stem cell through secondary spermatogonial stages of development. Interestingly, cadmium (Cd), an established mammalian spermatotoxicant, is taken up and retained preferentially in GZ/PrM regions of shark testis, where it increases the percentage of germinal clones undergoing apoptosis.

One mechanism by which Cd is reported to exert its toxic effects is by altering nuclear receptor-mediated gene transcription. To assess the utility of the shark testis model for identifying stage-related and toxicant-sensitive genes, the polymerase chain reaction-differential display (PCR-DD) method of mRNA fingerprinting was applied to tissues collected from control and Cd-injected animals. Poly (A+) RNA was prepared from staged tissues: GZ; PrM; meiotic (M; spermatocytes to early spermatids); and postmeiotic (PoM; elongating to mature spermatids). Five primer sets were used to obtain a total of 49 stage-dependent and 39 Cd-responsive bands. Three bands were subjected to further analyses: sequencing, 5' and 3' rapid amplification of cDNA

ends, reverse transcriptase-PCR, and Northern analysis. One differentially displayed band was highest in GZ/PrM stages, where it was upregulated fivefold by *in vivo* Cd treatment. It was identified as an approximately 400 base pair fragment of the control region of mitochondrial (mt) DNA, implying that Cd selectively enhances transcriptional activity on the H-strand early in spermatogenesis. Of the 12 proteins encoded in mtDNA, the cytochrome oxidase subunits have been implicated in caspase activation leading to apoptosis in somatic cells, and also are stage-dependent and androgen-responsive in rodent testis.

A second differentially displayed band (PoM > M > PrM/GZ) decreased after Cd treatment and ultimately was identified as the shark homolog of BRAP2 (Acc. #AF421550). BRAP2 is an evolutionarily conserved protein that regulates cytosol-to-nuclear shuttling of transcription factors and other nuclear proteins by specific binding to nuclear localization signals. A third PCR-DD band increased progressively from GZ/PrM through subsequent developmental stages and was downregulated by Cd. This band was identified as a shark-specific S100 subtype (Acc. #AF421551), one of a large family of Ca<sup>++</sup> binding proteins with diverse functions. Although functional studies are required, results obtained using Cd as an illustrative toxicant provide a starting point for uncovering spermatotoxic mechanisms and demonstrate the feasibility of PCR-DD as applied to the shark testis model for identifying toxicant-sensitive genes, processes, and stages of development.

## Mode and Mechanism of Action of the Chlorotriazine Herbicides

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The chlorotriazines (atrazine, simazine, etc.) are herbicides used extensively in the United States. Degradation by-products, as well as the parent compounds, have been detected in surface and groundwater in areas of major usage. Atrazine and simazine have been shown to cause an earlier onset of mammary tumors in Sprague-Dawley rats following long-term dietary exposure (400 ppm or approximately 22.5 mg/kg/day). A fundamental question concerning this effect was whether or not the tumors were induced by a direct action on the mammary gland tissue itself, or as a consequence of chlorotriazine-induced hormonal changes that create an endocrine environment that is conducive for mammary tumor growth. Because the majority of studies rule out a genotoxic mechanism for the development of mammary gland tumors, the primary focus of these studies was to identify the cascade of endocrine events leading to mammary tumor development.

These researchers found that the primary mode of action of these herbicides involves a disruption of the hypothalamic control of anterior pituitary function. Specifically, atrazine inhibits the pulsatile release of gonadotropin-releasing hormone (GnRH) and subsequent luteinizing hormone (LH) release. A similar decrease in prolactin (prl) secretion also was identified. Based on the understanding of the neuroendocrine alterations, it was found that the chlorotriazines and their primary metabolites disrupt ovarian cycles, cause full-litter resorptions, suppress suckling-induced prl release in the lactating dam, and delay puberty in juvenile male and female rats (no observed adverse effect levels and lowest observed adverse effect levels have been identified in all studies). The atrazine-induced suppression of suckling-induced prl release in the

lactating dam (birth to day 4) caused a significant increase in prostate inflammation (lateral lobes) in the adult male offspring.

A number of *in vivo* and *in vitro* studies were conducted to determine the mechanism(s) involved in the disruption of the hormonal control of these reproductive processes. *In vivo*, atrazine increases dopamine (DA) and decreases norepinephrine (NE) in the hypothalamus. These effects are consistent with the inhibition of the GnRH pulses and decreased LH and prl secretion. Using undifferentiated pheochromocytoma (PC12) cells, which constitutively synthesize DA and NE, this research found that the chlorotriazines (and metabolites) alter catecholamine metabolism, suggesting that these hypothalamic neurons represent one set of target cells. This project also is evaluating the potential involvement of aromatase, because changes in this enzyme may represent another cellular target for the chlorotriazines.

These studies provided a better understanding of the development of mammary gland tumors in rats and their relevance to humans. In brief, the ovarian condition induced in the female rat (persistent estradiol secretion) does not have a likely parallel in women, thus the significance of these tumors to humans is questionable. However, the changes observed in central nervous system and pituitary function raise new concerns about the potential health effects of these chemicals. Finally, the fact that the metabolites tested were similarly potent in inducing alterations in reproductive function underscores the need to take these metabolites into consideration in the overall assessment of the chlorotriazine herbicides.

## Gestational and Lactational Exposure of Male Mice to Diethylstilbestrol Causes Long-Term Effects on the Testis, Sperm Fertilizing Ability *In Vitro*, and Testicular Gene Expression

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The objective of this study was to determine the long-term effects of gestational and lactational exposure to diethylstilbestrol (DES) on testicular growth and histology, number of Sertoli cells, epididymal sperm count and motility, sperm fertilizing ability *in vitro*, and testicular gene expression using cDNA microarrays and real-time polymerase chain reaction (PCR) in B6D2F1 mice on postnatal day (PND) 21, 105, and 315. Pregnant females were gavaged daily with 0, 0.1, 1, or 10 µg DES in corn oil per kg of maternal body weight from gestational day 12 to PND 21. Male neonates were monitored for body weight and anogenital distance and weaned on PND 21. The testes from male offspring were examined on PND 21, 105, and 315 for changes in wet weight, histopathology, and number of Sertoli cells.

Epididymal sperm count, sperm motion parameters, and sperm fertilizing ability *in vitro* were measured on PND 105 and 315. There were no significant changes in testes weight, and histological examination of the testes revealed no treatment-related effects. However, stereological analysis of the testes indicated a significant decrease in the number of Sertoli cells per testis in the high-dose group, which persisted from PND 21 to PND 315 ( $p < 0.01$ ). Sperm count also was decreased in the high-dose group, but the decrease was only significant

on PND 315 ( $p < 0.05$ ). The number and percent of motile sperm and sperm velocity, linearity, and amplitude of lateral head displacement were unaffected. By contrast, *in vitro* fertilizing ability of epididymal sperm was significantly decreased in the high-dose group on both PND 105 ( $p < 0.001$ ) and PND 315 ( $p < 0.05$ ). Microarray analysis and confirming studies with real-time PCR identified early and latent alterations in the expression of genes involved in estrogen signaling (ER $\alpha$ ), steroidogenesis (SF-1, Cyp17, Cyp11a, Star, SR-B1), lysosomal function (LGP85, Psap), and regulation of testicular development (Tr2-11, Inhbc, Hoxa10).

The results demonstrate that early exposure to DES causes long-term adverse effects on testicular development and sperm function, and that these effects are associated with changes in testicular gene expression, even long after the cessation of DES exposure. These results also suggest multiple mechanisms by which early developmental exposure to estrogen disrupts estrogen signaling, steroidogenesis, Sertoli cell function, and testicular development. Subsequent studies also have revealed that gestational and lactational exposure to the phytoestrogen genistein does not induce the same spectrum of physiological and molecular effects in the testis as DES, thus suggesting that genistein acts through a distinct mechanism of action in the testis.

## Development, Application, and Validation of a Sheepshead Minnow Estrogen-Responsive cDNA Macroarray

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This presentation provides an overview of research conducted by the Gulf Ecology Division investigating the effects of endocrine-disrupting chemicals on estuarine fish species. A series of research studies were initiated to examine the comparative dose-response characteristics and potencies of estrogenic chemicals using the sheepshead minnow (*Cyprinodon variegatus*) as a small fish model. These studies required the development of procedures for measuring hepatic vitellogenin (VTG) mRNA synthesis and serum VTG levels in male sheepshead minnows in response to aqueous exposure to natural, pharmaceutical, and xeno-estrogenic chemicals. The time-course of hepatic VTG mRNA regulation and VTG plasma accumulation and clearance kinetics also were determined to add a temporal component to the field application and interpretation of VTG as a biomarker of exposure. In further studies, differential display techniques were applied to samples taken from dose-response studies to discriminate variably

expressed gene fragments between untreated control fish and fish treated with 17 $\beta$ -estradiol. Information from these studies was used to develop an estrogen-responsive cDNA membrane macroarray. Laboratory validation of the macroarray was accomplished by measuring the hepatic expression of these genes in male sheepshead minnows using fish previously exposed to 17 $\beta$ -estradiol, 17 $\alpha$ -ethynyl estradiol, diethylstilbestrol, methoxychlor, and p-nonylphenol. Identical patterns of gene expression were observed between native ligand, 17 $\beta$ -estradiol, and the four estrogenic compounds tested. In addition, intensities of the gene responses as measured on the macroarrays clearly followed a dose-response pattern for each chemical tested. The research described is the first step toward developing a suite of specific macroarrays for application to chemical screening and prioritization programs, or as a monitoring tool to identify chemical contamination of aquatic environments.

## Interaction of Estrogen and TCDD in an Avian Model

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This research group hypothesized that interactions between specific xenobiotics and endogenous factors that control cell function and differentiation can explain both similar and disparate toxic responses observed in nonmammalian species compared to laboratory models. Results from a series of studies support this hypothesis and show that the response of tissues from chickens respond differently to dioxin than do tissues from mammals. Furthermore, these studies indicate that the differences are largely attributable to the interaction between dioxin growth factors and hormones, including sex steroids. Previous studies showed that in rodents, males demonstrate a greater sensitivity to dioxin-induced decreases in body weight than females, and that this difference can be reduced by exogenous estrogen treatment. In contrast, immature female chickens were more sensitive than males, and estrogen treatment of immature male chickens replicated the increased sensitivity of females. Taken together, these studies suggest that some toxicants and estrogens act in concert at the level of the cell as modulators for cell growth and differentiation, energy homeostasis, intermediary metabolism, and lipid mobilization in both mammalian and avian species. More importantly, the results from these studies help explain how the mechanisms by which some toxicants interact with endogenous hormones, modulate targeted physiologic processes, and elicit adverse effects in a manner that is species-, gender-, and life-stage specific.

Estrogen treatment of male birds resulted in qualitatively similar lipid profiles to those of mature laying hens and estrogen-treated immature hens, thus providing a model by which to study dioxin-estrogen effects on lipid metabolism in the absence of the energetic needs of egg production. Resulting data show that dioxin antagonized several effects of exogenous estrogen in male chickens, and estrogen enhanced 2,3,7,8-tetrachlorodibenzodioxin

(TCDD) toxicity in a tissue-specific manner. Birds treated with estrogen alone had increased total triacylglyceride concentrations with specific increases in the  $\Delta 9$ -desaturase products 16:1n7, 18:1n7, 18:1n9, and 20:1n9, although these increases did not occur for birds treated with TCDD alone or in combination with estrogen. TCDD and estrogen plus TCDD treatments increased phospholipid concentrations of the diet-derived polyunsaturated fatty acids 18:2n6, 18:3n6, 20:3n6, 18:3n3, and 20:5n3, although only the estrogen plus TCDD group had significantly increased total phospholipids. TCDD and estrogen plus TCDD treatments decreased total concentrations of  $\Delta 9$ -desaturase products and saturated fatty acids, and estrogen treatment alone more specifically decreased concentrations of several saturated and polyunsaturated fatty acids. These findings support the hypotheses that differences exist in the response of different species, age-stage, and genders to the same toxicant.

As expected, the interaction of dioxin with estrogen was common to both mammals and birds, but unexpectedly, the nature of the interaction in mammals and birds was quite different. The protective effects of estrogen observed in mammalian species was not observed in the chicken model, and estrogen treatment actually augmented the adverse effects of dioxin in some avian tissues. The augmenting effect of estrogen on the metabolic effects of dioxin in chickens is consistent with a more severe effect of these compounds on reproduction in nonmurine vertebrates and may explain the failure of laboratory experiments utilizing rodent models to fully replicate adverse effects observed in the field. More importantly, the specific adverse effects of dioxin on key lipid mobilization may explain the wide range of developmental defects that are observed in egg-laying species compared to the effects observed in eutherian species.

## Masculinization of Invertebrates by Endocrine Toxicants: Mechanisms and Environmental Significance

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Field observations and laboratory studies have suggested that members of many invertebrate phyla are masculinized as a consequence of exposure to environmental chemicals. The overall objectives of this research program are to elucidate endocrine processes in a mollusk (*Ilyanassa obsoleta*) and in a crustacean (*Daphnia magna*) that control masculinization and identify mechanisms by which environmental chemicals can disrupt these processes.

The biocide tributyltin (TBT) has caused a pseudo-hermaphroditic condition (imposex) in many marine snail populations. The generally viewed mechanism by which TBT causes imposex is by inhibiting the metabolic conversion (via aromatase) of testosterone to 17 $\beta$ -estradiol. Experiments were conducted to establish the relationships among TBT exposure, elevated testosterone levels, and imposex. Exposure of female mud snails (*I. obsoleta*) to environmentally relevant concentrations of TBT significantly elevated both testosterone levels and the incidence of imposex. No evidence that elevated testosterone levels were due to the inhibition of testosterone aromatization was found. Rather, it was discovered that testosterone is extensively converted to fatty-acid esters in this species, which serves to maintain homeostasis of testosterone. TBT suppressed the esterification of testosterone, resulting in the elevation of free testosterone levels. Field studies demonstrated that fatty esterification is the major determinant of testosterone levels during the reproductive cycle of the snail, and that TBT-induced imposex populations of

mudsnails have compromised testosterone-fatty acid esterification capabilities.

Having established testosterone homeostasis as a target for the endocrine disruption in the snail, experiments were conducted to identify possible masculinizing effects of testosterone in the crustacean (*D. magna*). Exposure of maternal daphnids to testosterone had no effect on the sex of offspring. Rather, testosterone caused significant developmental abnormalities in progeny. Detailed analyses revealed that the developmental toxicity associated with testosterone was primarily due to anti-ecdysteroidal activity of this compound. The fungicide fenarimol also was found to elicit significant anti-ecdysteroidal activity. Ongoing studies suggest that 4-nonylphenol, propiconazole, and piperonyl butoxide also elicit antiecdysteroidal activity.

An extensive investigation was performed in search of a hormone that functions as a sex determinant in daphnids. This group of investigators discovered that the terpenoid hormone methyl farnesoate programs oocytes to develop into male progeny. Further, the insecticides methoprene and pyriproxifen were found to mimic methyl farnesoate and alter sex ratios of daphnid progeny. Results from this program have significantly advanced understanding of the endocrinology of masculinization in invertebrates and have identified susceptible targets of endocrine toxicants that can significantly alter sex ratios, sexual development, and fecundity of these organisms.

## Dioxin (TCDD) Disrupts Steroid Action in an Endometriosis Model

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Endometriosis is defined as the growth of endometrial glandular epithelium and stroma at an extra-uterine or ectopic site. Ectopic implantation of endometrial tissue entering the peritoneal cavity via retrograde menstruation requires an invasive event and the biomolecules necessary for establishment of endometriosis include the matrix metalloproteinases (MMPs). The MMPs are expressed in a cycle-dependent fashion, broadly expressed during menstruation and focally expressed during estrogen-mediated growth. These enzymes are largely absent during the progesterone-dominated secretory phase of the menstrual cycle, and their expression is inhibited by progesterone treatments *in vitro*. Ectopic endometriotic lesions have a decreased ability to respond to progesterone, impairing the therapeutic benefit of progestins; nevertheless, pregnancy can reduce the risk or impact of the disease in some women. A recent finding in this laboratory suggests that women with endometriosis have a decreased sensitivity to progesterone, which is associated with an increased expression of MMPs during the menstrual cycle. This research project explored whether environmental agents might impact the cellular mechanism(s) controlling endometrial MMP expression in women with endometriosis.

The spontaneous development of endometriosis in primates has been associated with experimental exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), although whether exposure to TCDD affects the establishment or progression of this disease in women remains unclear. To approach this question, culture models were examined in concert with an experimental model of endometriosis us-

ing human tissue and nude mice. Specifically, this research group examined if TCDD might impact endometriosis by disrupting normal steroid-mediated endometrial MMP expression. Initially, it was demonstrated that the ability of progesterone to suppress the expression and secretion of MMPs is reduced following the *in vitro* exposure of human endometrium to TCDD. *In vitro* treatments of human endometrium with TCDD subsequently blocked the ability of progesterone to prevent establishment of experimental endometriosis, following injection of tissue into nude mice. Further studies indicated that TCDD treatment of endometrial tissue *in vitro* is associated with a failure of progesterone to induce TGF- $\beta$ 2, a growth factor necessary for normal MMP regulation.

This project's most recent studies have demonstrated that TCDD further impairs progesterone action in endometrial tissue by suppressing progesterone receptor expression. Using stromal-epithelial co-cultures, it was found that TCDD exposure activates an epithelial-dominant pathway that increases the expression of MMPs through pro-inflammatory cytokine-mediated decreases in progesterone receptor. Together, these findings suggest that TCDD may influence the development of human endometriosis by decreasing endometrial sensitivity to progesterone. By several cell-specific mechanisms, TCDD acts to increase endometrial MMP expression, leading to increased invasive potential in an experimental endometriosis model. Current efforts seek to explore whether the molecular regulation of MMPs might be disrupted neonatally, prior to the development of active disease in adults.



## Chemical Interference With Non-Genomic Steroid Actions: A Novel Mechanism of Endocrine Disruption

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There is now convincing evidence that, in addition to the classic genomic mechanism of steroid action via binding and activation of nuclear steroid receptors, steroids also act at the cell surface of target tissues to initiate rapid, nongenomic responses, and that these actions are mediated by steroid membrane receptors. Steroid membrane receptors and rapid steroid actions, including activation of intracellular signaling pathways, have been identified in many tissues, including cardiovascular tissues, brain, pituitary, bone, kidney, liver, gonads, and gametes. Several recent studies have shown that nongenomic steroid actions, like genomic ones, are susceptible to interference by xenoestrogens. The project goals are to determine the mechanism, extent, and potential environmental hazards of this novel type of endocrine disruption. Recent studies with spotted seatrout and Atlantic croaker have shown that the mechanism of interference with these nongenomic steroid actions involves xenoestrogen binding to the steroid membrane receptors thought to mediate these actions. A variety of xenoestrogens, including kepone and o,p'-DDD, bind to the oocyte progesterin membrane receptor (mPR) in spotted seatrout and also antagonize progesterin-induced oocyte maturation in an *in vitro* bioassay at concentrations of  $10^{-6}$  to  $10^{-7}$  M (equivalent to 20-40 ppb, a tissue concentration frequently reported in fish from contaminated environments). Moreover, these xenoestrogen effects on receptor binding and oocyte maturation *in vitro* are reversible (i.e., they were not nonspecific toxic effects of the compounds), and both activities were completely restored after washing. Xenoestrogens also have been shown to interfere with the nongenomic action of progestins to increase sperm motility in these two fish species by binding to their mPRs on sperm.

The finding that the inhibitory action of a hydroxylated polychlorinated biphenyl (PCB) on sperm motility was partially reversed by addition of excess progesterin is consistent with a receptor-mediated mechanism of xenobiotic action.

Initial experiments show that the binding of several xenoestrogens (methoxychlor, hydroxylated PCBs, and dichlorodiphenyltrichloroethane derivatives) to the oocyte mPR is dependent on localization of the receptor in the plasma membrane and is related in part to their lipophilicity. These preliminary results suggest that xenoestrogen interactions with plasma membrane and nuclear steroid receptors are qualitatively different, and that membrane receptor-mediated steroid actions may be especially susceptible to interference by lipophilic xenobiotic compounds. Similar experiments will be conducted with the mPR on sperm and a membrane estrogen receptor in testes to confirm the broad applicability of these findings.

The current lack of information on the structures of any steroid membrane receptors and their steroid binding sites has prevented the development of experimental and theoretical approaches to determine their potential interactions with xenoestrogens at the molecular level. This research group recently cloned, sequenced, and characterized the seatrout oocyte mPR, the first steroid membrane receptor whose structure has been determined in any vertebrate species. The seatrout mPR has seven transmembrane domains, which is characteristic of G-protein-coupled receptors (GPCRs). Fourteen similar genes have been identified and partially characterized in other vertebrates, including three in humans. Preliminary results indicate that the recombinant proteins produced in a bacterial expression system transfected with the three human genes also bind progestins and have characteristics typical of mPRs. In conclusion, a new family of steroid receptors has been discovered that is structurally unrelated to nuclear steroid receptors, but instead has characteristics typical of GPCRs. Moreover, evidence has been obtained that xenoestrogens can interact with mPRs belonging to this receptor family. The nature of xenobiotic interactions with both the seatrout and human mPRs will be investigated at the molecular level.

## Environmental Androgens and Anti-Androgens

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Androgens play decisive roles in sexual differentiation of the gonads and accessory reproductive tissues during prenatal and neonatal development. In addition, androgens influence the acquisition and maintenance of secondary sex characteristics in adults. This research focuses on the impact that endocrine-disrupting chemicals (EDCs) can have on androgen action. This research integrates *in vitro*, *ex vivo*, and *in vivo* studies to provide mechanistic and dose-response information for risk assessment on EDCs. EDC action can seriously alter reproductive development when administered during critical, sensitive life stages. *In utero*, perinatal, and pubertal exposures to EDCs can produce severe effects on both male and female offspring, depending upon the chemical and its mechanism of action.

Several classes of chemicals have been studied that act as anti-androgens either by binding to the androgen receptor and interfering with normal androgen action or by inhibiting the synthesis of testosterone (e.g., linuron, ketconazole, vinclozolin, procymidone, dichlorodiphenyltrichloroethane and its metabolites, and phthalate esters). Recent work also includes the study of environmental samples and chemicals with androgenic activity such as 17 $\beta$ -trenbolone (TB), an anabolic steroid used as a growth promoter in beef cattle. Studies with TB have been conducted in both *in vitro* and in

short-term *in vivo* screening assays. In the rat *in utero* screening assay, maternal TB administration increased anogenital distance and attenuated the display of nipples in female offspring in a dose-related manner similar to the published effects of testosterone propionate. Previous studies have documented that these types of malformations in newborn and infant rats are not only permanent effects, but also are highly correlated with serious reproductive malformations in adults.

As tools in this research, several *in vitro* assays have been developed and utilized both for screening chemicals and as aids for defining mechanism of action. For example, cell lines have recently been developed that stably express either androgen- or estrogen-responsive luciferase-reporter genes. This research primarily utilizes steroid hormone receptors from mammalian species such as rat or human androgen receptors. It is assumed, but largely unproven, that EDCs will bind steroid receptors from mammalian and nonmammalian species with similar affinity. To test this hypothesis, this group also is attempting to identify, isolate, and sequence androgen and estrogen receptor proteins from several nonmammalian species. If time permits, there also will be a description of plans to incorporate these receptors into *in vitro* assays and assess receptor binding to EDCs across several species.

# **Poster Presentations**

## **Screening and Testing Assays**

## Computational Models for the Rapid Prediction of Ligand Binding Affinities to the Androgen Receptor: Effects of Mutations on Ligand-Receptor Specificity

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Classically considered the male sex hormone, dihydrotestosterone is critically involved in numerous physiological processes. In addition to initiation of male sexual differentiation and development of male secondary characteristics, dihydrotestosterone has been implicated in blood pressure regulation, obesity, and bone development in conjunction with estrogen. For the past several years, there has been growing concern regarding the influence of both natural and synthetic chemicals in the environment on normal physiological processes of both wildlife and humans as mediated by the androgen receptor (AR). Although the full impact of these chemicals on human health is still uncertain, it is evident that both basic and applied research into this issue is essential. In this present study, the binding affinities of a series of compounds for both the rat and human AR were estimated from calculated ligand-receptor binding energies. A strong correlation ( $r^2 = 0.76$ ) was found between the computed binding energies for this series of compound and the corresponding published values of the observed relative binding affinity for rat AR. When these values of the bind-

ing energy were included as an additional descriptor to build three-dimensional quantitative structure-activity relationship (3D-QSAR) models using comparative molecular field analysis, the predictive ability of the models was improved dramatically for a series of external test-set compounds not employed for model building. These 3D-QSAR models currently are undergoing further testing to determine their utility in screening chemical libraries for endocrine-disrupting compounds that preferentially bind the AR.

Similar binding energy calculations were repeated for a particular AR variant containing the single-site Thr877Ala mutation, which has been associated with failure of hormonal therapy in the treatment of human prostate cancer. Comparison of ligand-binding energies revealed a general trend of enhanced binding affinity for this AR variant over the wild-type AR, consistent with published experimental findings that this AR variant exhibits reduced ligand specificity and is inappropriately activated by progestins, estrogen, and even the anti-androgen hydroxyflutamide.

## **A Computer-Docking Study of the Binding of Polycyclic Aromatic Hydrocarbons and Their Metabolites to the Ligand-Binding Domain of the Estrogen Receptor**

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Polycyclic aromatic hydrocarbons (PAHs) are a class of ubiquitous, anthropogenic chemicals found in the environment. In the present study, computational methods are used to evaluate their potential estrogenicity and the contribution that chemicals in this class make to environmental estrogenicity. Classical docking methods, dock and affinity, are used to evaluate the potential binding affinity of chemicals in this class and their metabolites to the published crystal structures of the ligand-binding domain of the estrogen receptor. These methods, with a molecular mechanics interaction energy scoring function, were able to place estradiol within a root mean square deviation of 0.4 Å from its binding position determined by x-ray crystallography. The scores obtained in this manner show wide variation for the PAHs and their metabolites. They depend on PAH type and the three-dimensional structure of the metabolites. For example (-)-antibenzo-

[c]phenanthrene diolepoxide is a much better binder in this model than the (+) enantiomer. A few dominant modes of binding have been identified and will be presented. These results will be compared to the results for known binders. Semi-empirical quantum mechanical methods also were used to compute the interaction energy of the most stable structures obtained from the classical computer-docking experiments. These quantum mechanical calculations provide a quantitative description of the interaction between the ligand and the receptor, and contain elements that are omitted from the classical scoring function. The comparison of these results demonstrates the importance of the nonclassical interaction terms for molecules that have pi electron systems. K.W. Brown was funded by EPA/UNC Toxicology Research Program Training Agreement CT902908 and CT827206 during the performance of this study.

## Rainbow Trout Androgen Receptor Alpha and Human Androgen Receptor: Comparisons in the COS Whole-Cell Binding Assay

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Typically, *in vitro* hazard assessments for the identification of endocrine-disrupting compounds (EDCs), including those outlined in the Endocrine Disruptor Screening and Testing Advisory Committee Tier 1 Screening protocols, utilize mammalian receptors. However, evidence exists that fish sex steroid hormone receptors differ from mammalian receptors both structurally and in their binding affinities for some steroids and environmental chemicals. Most of the binding information available to date has been conducted using cytosolic preparations from various tissues. This research project sought to compare competitive binding using rainbow trout androgen receptor alpha (rtAR) and human androgen receptor (hAR) expressed in transfected COS (African green monkey kidney) cells. In this system, the binding affinities of individual receptors can be investigated without the potentially confounding effects of other steroid receptors present in cytosolic tissue extracts. Saturation ligand binding and Scatchard analysis using [<sup>3</sup>H]R1881, a synthetic androgen, revealed a dissociation constant ( $K_D$ ) of 0.24 nM for the rtAR. In the same system, a  $K_D$  of 2.27 nM was found for the hAR. Binding studies in

competition with [<sup>3</sup>H]R1881 were conducted using steroids and a selection of environmental chemicals shown to bind mammalian AR.

All of the chemicals and steroids studied competed for binding in both rtAR and hAR. The relative order of binding affinities of natural and synthetic androgens for the rtAR was methyltrienolone > trenbolone > 11-ketotestosterone > dihydrotestosterone (DHT) > testosterone > androstenedione. The rank order for the hAR was similar, except that DHT and testosterone had higher affinity than 11-ketotestosterone. Also, it was found that androstenedione bound with lower affinity than what has been reported in the literature by Wells and Van Der Kraak for the rtAR. Other steroids and anti-androgens, such as progesterone, 17 $\beta$ -estradiol, hydroxyflutamide, vinclozolin and its metabolites M1 and M2, and 2,2-bis-(4-chlorophenyl)-1,1-dichloroethene also were studied, and their relative binding order was similar for the two species. Studies such as these will facilitate the identification of EDCs that affect many species and support future risk assessment protocols.

## Semiquantitative Laser-Scanning Confocal Microscopy (LSCM): Assessing Crustacean Egg Quality for EDC Screening

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Laser-scanning confocal microscopy (LSCM) is widely used by biomedical investigators, but its application to environmental toxicology, in particular marine ecotoxicology, is practically nonexistent. A new semiquantitative LSCM approach is described here for assessing relative yolk quantity in marine invertebrate embryos (harpacticoid copepods) after rearing of their parents in polycyclic aromatic hydrocarbons (PAHs). These researchers found that LSCM represents a powerful, easy, but largely unexplored ecotoxicological tool for rapidly assessing *in vivo* effects of endocrine-disrupting chemicals (EDCs) on crustacean embryo quality and development.

In this study, the common PAH chrysene (CHRY) was selected as a model toxicant to investigate the utility of the lipovitellin-based LSCM egg/embryo quality screening tool. CHRY has a chemical structure that is steroidal in nature, particularly when photo-oxidized by ultraviolet (UV) light to 6-hydroxychrysene. This photo-oxidation product exhibits anti-androgenic properties *in vitro* in mammalian models, and may have endocrine-active properties in marine invertebrates. In this study, it was hypothesized that vitellogenesis may be affected in female copepods (*Amphiascus tenuiremis*), and that these effects could be detected in 1 day-old embryos via fluorogenic labeling with the yolk-specific probe BODIPY® 505/515 and direct LSCM photomultiplier-based measurement.

The fluorescent yolk-labeling method described here was able to stain and detect statistically significant differences in yolk concentrations in *A. tenuiremis* eggs from females exposed to UV and/or CHRY-contaminated sediments. Control yolk intensities in less than 24-hour-old embryos of females cultured throughout their lifecycle in clean sediments were statistically identical with or without UV exposure. In contrast, yolk intensities in less than 24-hour-old embryos of females cultured throughout their lifecycle in CHRY-contaminated sediments were significantly higher in the non-UV exposed 2,500 ng CHRY/g-sed (67% higher) and UV-exposed 500 ng CHRY/g-sed (76% higher) treatments. Females exposed to 500 ng CHRY/g-sed without UV exhibited yolk intensities that were significantly lower (18%) than UV-exposed females at the same CHRY concentration, but significantly higher (45% higher) than both UV and non-UV exposed controls. A fivefold increase in CHRY concentration (2,500 ng CHRY/g-sed) in the absence of UV also enhanced yolk deposition to eggs, but yolk levels were modestly (but significantly) lower (6%) than eggs from the UV-exposed 500 ng CHRY/g-sed treatment. CHRY exposure during maturation to female reproductive maturity significantly enhanced yolk deposition to eggs/embryos and was strongly enhanced by UV irradiation. Although the direct mechanism of CHRY-induced yolk deposition is unknown, CHRY may exhibit hormonal properties that mimic endogenous crustacean hormones such as ecdysteroids.



## Determination of Structural Requirements for Activation of the Clearance Mechanism of Environmental Pollutants and Xenobiotic Chemicals by the Pregnane Xenobiotic Receptor: Species-to-Species Extrapolation

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Increases in the prevalence of certain cancers (e.g., breast, prostate, testicular, ovarian) may be related to interactions between components of the endocrine system and environmental molecules known widely as “endocrine-disrupting chemicals.” However, there is uncertainty as to the mechanism of the effects induced by a particular compound and the dose at which the effect is elicited as a direct result of exposure. There is equal uncertainty as to what degree the data from wildlife and laboratory animal model systems can be extrapolated to measure the risk of human exposure to the same xenobiotic chemicals. Recent evidence shows that an “orphan” nuclear receptor known as the pregnane xenobiotic receptor (PXR) plays a key role in regulation of gene expression of cytochrome P450-3A (CYP3A) proteins that metabolize a wide variety of chemicals including toxins, environmental contaminants, and endogenous compounds such as toxic bile acids and steroids. Elucidation of the structural features of PXR required for binding of diverse classes of harmful endogenous and exogenous compounds is crucial to understand the clearance pathways and predict (and thereby avoid) dangerous interactions with the endocrine system. The objective of the present study is to employ computer-aided molecular modeling strategies as a means of offering guidance into the molecular basis for commonalities and differences in how humans and model animals respond to chemical exposure as mediated by PXR.

Computer-based approaches are proving useful in risk assessment to help prioritize existing chemicals in terms of their endocrine-disrupting effects prior to labor-intensive and time-consuming *in vitro* and *in vivo* biological testing. Such approaches also are valuable for predicting the potential endocrine-disrupting activity of new chemicals before they enter the environment. Comparative molecular field analysis (CoMFA) and related approaches have been used to develop three-dimensional quantitative structure-activity relationship (3D-QSAR) models for several compound data sets: diindolylmethanes, clotrimazole analogues, polychlorinated biphenyls, and a combined set of receptor agonists that includes soil contaminants and toxins. Computational homology modeling techniques were employed to construct a hypothetical 3D structure of mouse PXR (mPXR from its human orthologue, hPXR). Examples will be presented that demonstrate the utility of the hPXR crystal structure and the mPXR homology model in screening small-molecule ligands for prediction of their potential harmful effects on the endocrine system, either rapidly using “docking and scoring” routines or more rigorously using calculated ligand-receptor binding energies. QSAR models also have been developed for these data sets employing two novel computational tools, the polynomial neural network (PNN) and the volume learning algorithm (VLA). The PNN and VLA exhibit superiority over traditional QSAR techniques in terms of their predictive ability.

## Utility of *In Vitro* Assays To Screen in Environmental Mixtures for EDC Activities

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Female mosquitofish (*Gambusia affinis holbrooki*) downstream from Kraft paper mills in Florida display masculinization of the anal fin, an androgen-dependent trait. The first series of studies were designed to determine if water contaminated with pulp mill effluent (PME) from the Fenholloway River, FL, displayed androgenic activity *in vitro* and to relate this activity to the reproductive status of female mosquitofish taken from this river. Eighty percent of the female mosquitofish from the Fenholloway River were partially masculinized, while another 10 percent were completely masculinized based on the number of segments in the longest anal fin ray ( $18.0 \pm 0.4$  versus  $28.1 \pm 0.9$  [ $p < 0.001$ ]) in a control river versus Fenholloway River, respectively. In a COS whole-cell binding assay, all three PME samples displayed affinity for human androgen receptor (hAR) ( $p < 0.001$ ). In addition, PME induced androgen-dependent gene expression in CV-1 cells (cotransfected with pCMV hAR and mouse mammary tumor virus [MMTV] luciferase reporter), which was inhibited by approximately 50 percent by coadministration of hydroxyflutamide, an AR antagonist. When CV-1 cells were transfected with human glucocorticoid receptor rather than hAR, PME failed to significantly induce MMTV-luciferase expression. Further evidence of the androgenicity was observed using a COS cell AR nuclear translocalization assay. PME bound hAR and induced translocalization of AR into the nucleus. PME also displayed “testosterone-like” immunoreactivity in a testosterone radioimmunoassay, whereas water from the reference sites did not. In summary, water collected downstream of the Kraft mill on the Fenholloway

River contains unidentified androgenic substances whose presence is associated with masculinization of female mosquitofish. Currently, PME samples are being fractionated in attempt to identify the androgenic chemicals.

In a second project, androgenic activity was detected in feedlot effluent from a feedlot in the Midwestern United States. However, it has not yet been determined if this activity arises from natural or synthetic hormones. The potency of beta trenbolone also was examined for androgenicity. Trenbolone is an anabolic steroid used to promote growth in beef cattle found in feedlot waste water and manure samples. Based on observations of reproductive alterations in fish in waters receiving feedlot effluent, concern has arisen about the presence and persistence of this hormonally active substance in effluent-reaching streams near the feedlots. *In vitro*, beta trenbolone was a full agonist, approximately as active as dihydrotestosterone, and this activity was fully inhibited by the antiandrogen hydroxyflutamide. When examined *in vivo* in the Hershberger Assay, trenbolone displayed selective androgenic receptor-mediated (SARM) activity, affecting some androgen-dependent tissues much more than others in a manner suggesting that 5-alpha-reduction inactivated rather than activated the parent compound. In summary, trenbolone is a potent SARM. Further studies are in progress to: (1) determine whether trenbolone is present in feedlot effluent in concentrations sufficient to induce effects, and (2) characterize the ability of this chemical to alter vertebrate reproduction and development.

## Endocrine Disruptors From Combustion and Vehicular Emissions: Identification and Source Nomination

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During the last decade, concerns have been raised regarding the possible harmful effects of exposure to certain chemicals that are capable of modulating or disrupting the function of the endocrine system. These chemicals, which are referred to as endocrine-disrupting chemicals (EDCs), have the capability to interfere with the production, release, transport, metabolism, or elimination of the natural hormones in the body responsible for the regulation of developmental processes. Recently, exhaust samples from combustion and vehicular sources

are being analyzed to provide initial identification of EDCs. The intent of this screening effort is to provide discerning evidence for nominating sources for further EDC characterization. Conventional sampling, advanced analytical methods, and bioassays are being used to provide initial characterization of these samples for their compound identity and EDC activity. The intent of this research is to sample and chemically characterize multiple combustion sources to determine whether EDCs are emitted from combustion sources and in what quantity.

## Endocrine-Mediated Effects of UV-A Irradiation on Grass Shrimp (*Palaemonetes pugio*) Reproduction: Implications for EDC Screening

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Little is known concerning the interaction between ultraviolet (UV) light and endocrine-disrupting chemical (EDC) exposure on crustacean reproductive cycles. Perturbations to the relationships between reproduction and UV light in laboratory environments may induce responses that are attributed incorrectly to toxicant exposure. In this study, male and female grass shrimp, *Palaemonetes pugio*, were exposed to sublethal concentrations of endosulfan (ES; 200 ng/L and 400 ng/L) under both white fluorescent (WF) and UV-A (315–400 nm) light conditions for 50 days in laboratory bioassays.

Female endocrine (vitellogenin, ecdysteroids, and cholesterol), reproductive (percent gravid, clutch size), and embryo (days to hatch, hatching success, and hatching survival) responses were assessed. UV exposure alone caused a significant (more than fourfold) increase in total *P. pugio* female egg production over the course of 50 days. Exposure to ES and UV light significantly lowered the percentage of gravid females relative to UV controls, whereas ES-exposed shrimp under WF lighting did not exhibit these trends. Although higher vitellogenin concentrations and lower ecdysteroid titers were correlated with increased female egg production, cholesterol titers only exhibited a dose-dependent change when exposed to ES. Embryos

from females exposed to UV light had significantly lower ecdysteroid titers and shorter hatching times, but there were no differences in embryo vitellogenin concentrations, hatching success, or hatching survival. These results indicate that UV-A exposure has a pronounced effect on grass shrimp (*P. pugio*) reproduction and likely is mediated through 5-hydroxytryptamine-related neuroendocrine pathways.

This project showed that UV-A radiation alone can significantly alter chronic endpoints (e.g., reproduction, egg quality, etc.) typically measured in crustaceans when evaluating xenobiotic effects. In this study, ES simply countered UV-mediated stimulatory reproductive effects and either: (1) induced shunting of female energy away from reproduction to xenobiotic metabolism, or (2) interacted with UV-triggered neurotransmitter signaling. Regardless, subtle ES effects were only detected under UV-A light. Because UV-A has a profound effect on grass shrimp reproductive biomarkers and responses, and because UV is an environmentally relevant parameter, pesticides with (e.g., fipronil) and without (e.g., ES) UV degradation potential should be evaluated under UV exposure. Furthermore, the definition and scope of endocrine disruptor research should be expanded to incorporate the complex interplay between environmental factors and multiple endocrine feedback processes.

## Enzyme-Linked Immunosorbant Assays for Detection of Crustacean Vitellin and Ecdysteroids: Development and Validation for EDC Screening

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Little is known regarding the effects of endocrine-disrupting chemicals (EDCs) on crustacean vitellogenesis and hormone synthesis. Recently, this laboratory developed simple, fluorescence-based enzyme-linked immunosorbant assays (ELISAs) for crustacean lipovitellin and ecdysteroid quantification in 96-well microplates. Although vitellogenin ELISAs are widely available for assessing exposure to environmental estrogens in fish, quantitative assays for crustacean vitellin/vitellogenin are lacking. Additionally, nonradiometric approaches for crustacean ecdysteroid quantification have previously been unavailable. These ELISAs involve: (1) competing reactions between antigens and vitellin- or ecdysteroid-specific antibodies, and (2) detection via enzyme-labeled conjugates and fluorescent substrates. Both assays take only 3 days from start to finish (approximately 100 samples) and exhibit a high degree of accuracy and reliability.

For the indirect, competitive vitellin ELISA, a vitellin standard was purified from grass shrimp (*Palaemonetes pugio*) embryos (Stage I-II) by immunoaffinity chromatography and lyophilized into 1 µg aliquots. This ELISA involves competing reactions between coated or free lipovitellin and crossreactive polyclonal anti-amphipod (*Leptocheirus plumulosus*) vitellin antibodies. After designing four different ELISAs, it was found that maximum sensitivity ( $\geq 150$  pg vitellin) and reliability ( $R^2 > 0.99$ ) was

approached by simply increasing the primary and secondary antibody incubation times. This vitellin ELISA yields a dynamic standard curve (2-1,000 ng/mL) and low intra- and interassay variability (less than 10%). Because vitellin was detected in several crustaceans (e.g., grass shrimp, amphipods, and copepods), this ELISA may be potentially useful for other marine crustaceans. The effects of the gamma-aminobutyric acid (GABA) disrupting insecticide fipronil on male and female copepod (*Amphiascus tenuiremis*) vitellin production were evaluated using this ELISA.

The indirect, competitive ecdysteroid ELISA involves competing reactions between free ecdysteroids, conjugates of 20-hydroxyecdysone (20HE) and horseradish peroxidase, and 20HE-specific polyclonal antibodies. This ecdysteroid ELISA yields a dynamic standard curve (1-200 femtomoles/well) and can be used for all crustacean species. This ELISA is not just specific for 20HE, as it also detects intermediate ecdysteroids such as ecdysone, 3-dehydroecdysone, and 25-deoxyecdysone. For assay validation, ecdysteroid concentrations were measured in various life-cycle and developmental stages of the marine benthic copepod *A. tenuiremis* and the amphipod *L. plumulosus*. Additionally, the effects of the GABA-disrupting insecticide fipronil on reproductively successful/unsuccessful male and female copepod (*A. tenuiremis*) ecdysteroid titers were evaluated using this assay.

## Reproductive Alterations in the Grass Shrimp (*Palaemonetes pugio*) Following Pesticide Exposure

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This study examined the effects of sublethal chronic pesticide exposure on the reproductive output of the grass shrimp, *Palaemonetes pugio*. Grass shrimp are an important ecological species in estuarine systems and are exposed to anthropogenic contamination through nonpoint source runoff. Grass shrimp larvae were reared in the laboratory until sexually mature and exposed to individual pesticides for between 30 and 40 days. The four pesticides evaluated were endosulfan (organochlorine insecticide), chlorpyrifos (organophosphorus insecticide), methoprene (insect growth regulator), and fipronil (phenylpyrazole insecticide). The concentrations for each exposure were as follows: 200 and 400 ng endosulfan/L, 100 and 200 ng chlorpyrifos/L, 1 mg methoprene/L, and 100 and 200 ng fipronil/L. Shrimp were removed from the exposure cham-

bers when visible clutches appeared on the abdomen and frozen for further analysis. Endpoints analyzed included the rate at which females became ovigerous in each population and the potential reproductive output based on average clutch sizes. Results indicated that endosulfan significantly reduced the rate at which gravid females occurred during exposure in a dose-dependant manner. Chlorpyrifos results suggested that there may be an effect on reproduction, although the data did not exhibit a dose-dependant trend. There was virtually no difference in the rate of ovigerous females occurring when shrimp were exposed to either fipronil or methoprene. Work is continuing to evaluate a variety of physiological endpoints, including quantification of vitellin, ecdysone, cholesterol, and total lipids.

# Influence of the Structural Diversity of Data Sets on the Statistical Quality of Three-Dimensional Quantitative Structure-Activity Relationship (3D-QSAR) Models: Predicting the Estrogenic Activity of Xenoestrogens

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Federal legislation has resulted in the two-tiered *in vitro* and *in vivo* screening of some 80,000 structurally diverse chemicals for possible endocrine-disrupting effects. To maximize efficiency and minimize expense, prioritization of these chemicals with respect to their estrogenic disrupting potential prior to this time-consuming and labor-intensive screening process is essential. Computer-based quantitative structure-activity relationship (QSAR) models, such as those obtained using comparative molecular field analysis (CoMFA), have been demonstrated as useful for risk assessment in this context. In general, however, the CoMFA models developed to predict estrogenicity have been constructed from data sets with limited structural diversity. In this study, CoMFA models were built based on biological data for a structurally diverse set of

compounds spanning eight chemical families. This research group also compared two standard alignment schemes employed in CoMFA, namely *atom-fit* and *flexible field-fit*, with respect to the predictive capabilities of their respective models for structurally diverse data sets. The present analysis indicates that *flexible field-fit* alignment fares better than *atom-fit* alignment as the structural diversity of the data set increases. Values of log RP, where RP is equal to relative potency, predicted by the final *flexible field-fit* CoMFA models, are in good agreement with the corresponding experimental values for this data set. By virtue of the structural diversity of the data set chosen to build them, these three-dimensional QSAR models should be effective for predicting the endocrine-disrupting potential of chemicals that span a range of chemical families.

## **Developmental Exposures**



## **Neurophysiological Consequences in Hippocampus as a Function of Developmental Hypothyroidism**

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Thyroid hormones are essential for maturation and function of the mammalian central nervous system. Severe congenital hypothyroidism results in irreversible structural damage and mental retardation in children. Although a variety of environmental contaminants have been demonstrated to alter circulating levels of thyroid hormones (e.g., polychlorinated biphenyls, brominated flame retardants, drinking water contaminants, pesticides), the neurotoxicological effects associated with such hormone reductions have not been adequately assessed. Thyrotoxicants such as propylthiouracil (PTU) have been used pervasively in studies designed to determine the role of thyroid hormone in brain development. Although it is well established that the hippocampus is a brain region impacted by hypothyroidism, functional assessment of the neurophysiological integrity of the hippocampus following such treatment is lacking. Moreover, little information is available on more modest perturbations in thyroid hormones that would mimic those induced by environmental agents.

This presentation will focus on recent data characterizing the physiological changes associated with developmental hypothyroidism induced by PTU. Synaptic transmission and plasticity in the dentate gyrus of the hippocampus of adult offspring of pregnant dams treated with A1254 and

PTU are clearly altered, but the pattern of change is distinct between these two types of treatment. The age and duration of exposure, the age and site of assessment, and the relative change in the two main thyroid hormones T3 and T4 all may contribute to differential patterns of effects observed. Differential effects also are evident between the CA1 and dentate subregions of the hippocampus. However, certain similarities also exist, a predominant feature being the irreversible nature of the developmental insult in a brain region critical for cognitive function. Exposure limited to the perinatal period produced alterations in synaptic transmission and plasticity in adulthood, despite elimination of the contaminant and a return of thyroid hormones to normal range.

Future work will extend these observations by characterizing more fully the dose-response relationships, examining behavioral correlates of altered physiology, and evaluating environmentally significant thyroid-disrupting chemicals including brominated flame retardants and perchlorate. Increased understanding of the long-term consequences of mild perturbation of thyroid hormones during brain development will aid in assessment of the potential health hazards posed by environmental contaminants that interfere with thyroid hormone function.

## Retinoids Induce All the Limb Malformations Observed in Wild Populations of Deformed Frogs

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There is considerable evidence that wildlife and domestic animals have suffered adverse consequences from exposure to environmental chemicals that interact with components of the endocrine system. To assess the significance of endocrine disruptors that activate retinoid-signaling pathways for their role in causing limb developmental deformities in frogs, and to understand their mechanism of action to assess their implications for human health, this research project focused on the effects of disruption of retinoid-sensitive signaling pathways. These studies demonstrated that all the malformations observed in wild populations of frogs can be induced by experimental exposure to retinoids.

The research focused on the effects of (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB), which specifically activates the retinoic acid receptor, and led

to the discovery that there are multiple developmental windows of sensitivity to retinoid exposure. The experimental phenotypes include normal development, early embryonic lethality, duplicated limb buds, bony triangles, and truncated limbs. These phenotypes could be elicited in response to treatment with retinol palmitate, atRA, and TTNPB. A notable result is that limb dysplasias were only observed when larvae were treated during specific stages of limb bud development (stages 50–52 in *Xenopus laevis*; see Table 1). Exposures at earlier developmental stages induced malformations in other organ systems (e.g., craniofacial, axial), but not in limbs. Treatment at all stages is developmentally toxic at high doses and long exposures, although an interesting observation is that TTNPB treatment is not acutely toxic when treatments are after the gastrula stage. The surviving larvae typically die a few weeks after exposure.

**Table 1.** Treatment of *Xenopus laevis* with TTNPB elicits stage-specific limb defects.

TTNPB	Stage 50		Stage 51		Stage 52	
	% mortality	% malformed	% mortality	% malformed	% mortality	% malformed
None	12.5	0	40	0	10	0
$8 \times 10^{-9}$	44.4	0	70	0	40	33.3
$4 \times 10^{-8}$	25	0	41.6	50	10	66.6
$8 \times 10^{-8}$	33.3	0	58.3	80	11.1	100
$4 \times 10^{-7}$	50	0	100	0	72.7	100
$8 \times 10^{-7}$	50	0	100	0	77	100

## Low Doses of Estrogenic Chemicals Like Diethylstilbestrol During Development Result in Permanent Alterations in the Reproductive Tract

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Adverse human health consequences may result from exposure to chemicals that interact with the endocrine system, as documented in experimental animals and wildlife. Because the developing organism is uniquely sensitive to perturbation by chemicals with hormone-like activity, the present study addresses whether exposure to these chemicals during critical stages of differentiation will permanently alter the developmental program of tissues, so that they respond atypically to further stimuli later in life. Outbred CD-1 mice were treated by subcutaneous injections with diethylstilbestrol (DES; 0001-1,000 µg/kg) dissolved in corn oil, or corn oil alone (control) on days 1-5 of neonatal life. Mice were weaned at 17 days prior to puberty, housed 4 per cage, and challenged with three daily doses of 17β-estradiol (500 µg/kg) or DES (10 µg/kg). On the fourth day, uterine weight-body weight ratios were determined. Uterine tissues were microscopically evaluated for changes in epithelial cell height and number, gland number, and induction of estrogen-responsive proteins, including lactoferrin, progesterone receptor, and c-fos. Neonatal DES exposure resulted in altered uterine

response to estrogen at puberty. Of particular interest was that the response varied depending on the dose of neonatal exposure; neonatal exposure to DES 0.01 caused an enhanced response to estrogen at puberty as compared to controls, whereas higher neonatal doses of DES caused reduced uterine response. To determine if these effects were permanent, an additional group of mice was neonatally treated with DES (0.001-10 µg/kg) and housed until 4-5 months of age. These adult mice were ovariectomized and challenged 7 days later as described for the immature mice. The adult mice exhibited similar results (enhanced uterine wet weight response at the 0.01 dose and a dampened response at the high doses of 1 and 10 µg/kg). Mechanisms responsible for this altered response involved ERα-mediated events. This research group concluded that altered uterine responses were permanently imprinted by developmental exposure to estrogens. Other environmental estrogens, including genistein, the naturally occurring phytoestrogen found in soy products, are being tested and compared to DES to determine if similar altered uterine responses occur.

## **Field Studies**

## Endocrine-Disrupting Chemicals and Thyroid Outcomes

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Each year, nearly 10 million Great Lake Basin residents consume Great Lake sport fish (GLSF). These consumers represent the largest, non-occupational exposure to persistent organic pollutants. Polybrominated diphenyl ethers (PBDEs) recently have been detected in Great Lakes fish. These compounds are structurally similar to polychlorinated biphenyls (PCBs), which have been shown to have negative effects on thyroid function. This study will examine the hypothesis that PDBEs may act separately or synergistically with PCBs to impair thyroid gland and reproductive hormone function. An existing, well-characterized cohort of 4,206 GLSF consumers will be the focus of this research.

Cohort members will be interviewed to assess current and historic fish consumption, dietary and potential occupational exposures to PBDEs and PCBs, and endocrine health status. Cohort members who have not been diagnosed with an endocrine disorder will be invited to donate blood and urine samples and complete a detailed questionnaire ascertaining medical, GLSF consumption, occupational, and dietary histories. Pollutants such as 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE), PCBs, PBDEs, as well as thyroid and reproductive hormones will be assayed. Hemoglobin A<sub>1</sub>C and total serum lipids also will be measured. The association of endocrine measurements with total and congener-specific pollutants and GLSF consumption will be examined. Interactive effects will be assessed.

A pilot study of this cohort showed inverse associations between PCB levels and T<sub>4</sub> and free thyroxine index (FTI).<sup>1</sup> Associations were stronger in women than

in men, with less consistent effects on T<sub>3</sub> and thyroid-stimulating hormone (TSH). In women, the association of PCB with FTI—but not with T<sub>4</sub> level—and in men the association with T<sub>4</sub> remained significant after adjustment for years eating GLSF. Among male subjects, inverse PCB associations were seen with testosterone and sex hormone binding globulin-bound testosterone after adjustment for fish consumption.

Steroid hormones were not measured in women. In women, the number of GLSF meals eaten during the last year was inversely associated with T<sub>4</sub> and FTI, and positively related to TSH, although only the association with FTI was significant after adjustment for PCB level. In men, TSH was negatively associated with GLSF consumption, but this association was not significant after adjustment for PCB level. DDE was not associated with thyroid hormone levels.

Prior studies, combined with data from this cohort, suggest that fish consumption is associated with lower levels of T<sub>4</sub> and FTI. In these preliminary data, the inverse associations of PCBs with T<sub>4</sub>, FTI, and SHBG-bound testosterone remained significant after adjustment for fish consumption. Conversely, the association of number of GLSF meals in the last year with FTI, but not T<sub>4</sub> in women, remained significant after control for PCB level, suggesting that there may be factors in fish, other than PCBs, that contribute to the thyroid effects.

PBDEs will be investigated in this study for the potential additive or interactive effects on endocrine function with other environmental pollutants.

<sup>1</sup>Persky V, Turyk M, Anderson HA, Hanrahan LP, Falk C, Steenport DN, Chatterton R, Freels S, and the Great Lakes Consortium. The effects of PCB exposure and fish consumption on endogenous hormones. *Environ Health Perspect* 2001;109:1275-83.

## Latent Effects of Gestational Exposure to Heptachlor

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This research will investigate whether gestational exposure to the chlorinated cyclodiene insecticide heptachlor permanently alters reproductive and immune function. The study is based on a well-characterized episode in which the entire commercial milk supply on the Island of Oahu in Hawaii was contaminated with heptachlor epoxide during a 15-month period (1981-82), resulting in gestational exposure to the offspring of women who drank cows' milk during that period.

The research project will evaluate two primary hypotheses: (1) reproductive function in young adults who were exposed to heptachlor during gestation will be deficient compared to a control group, and (2) immune system function will be altered in the heptachlor-exposed group compared to the control group. Secondary hypotheses are: (1) the biological indicators of reproductive and immune function will correlate with quantitative estimates of heptachlor exposure, and (2) there will be gender-specific changes in reproductive endocrine and immune function associated with the heptachlor exposure.

The study will assess biological indicators of reproductive and immune function in 400 young adults who were *in utero* on Oahu at the time of the milk contamination and have resided their whole lives on Oahu. In addition, 200 unexposed comparison participants matched for age and ethnicity will be studied, including 100 long-term residents of Oahu who were not born on the island and 100 native residents of neighboring islands in Hawaii who were not impacted by the milk contamination. The study will involve a representative population of young adults, born between July 1981 and June 1982, who participated in a recently completed study of neurobehavioral effects

of this exposure, plus additional young adults recruited from the neighboring islands.

The study will assess biological indicators of reproductive and immune function. Hypothalamic-pituitary-gonadal axis function will be assessed by measuring serum reproductive hormone concentration: testosterone in men, estradiol and progesterone in women, and luteinizing hormone and follicle-stimulating hormone in both men and women. In the men, semen samples will be obtained for determination of sperm quality. The women will be asked to collect daily first morning urine specimens and record menstrual histories for 6 weeks. Levels of luteinizing hormone, estrone-3-glucuronide, and pregnanediol 3-alpha-glucuronide will be measured in the urine. Cell-mediated (Th1) immunity will be evaluated by antibody titer response to immunization with tetanus and multivalent pneumococcal vaccine. The proportion of Th1 and Th2 type CD4+ cell subsets in peripheral blood will be assessed using *in vitro* analysis of cytokine expression following activation. Susceptibility of peripheral blood T cells to activation-induced cell death will be assessed using *in vitro* analysis of Fas (CD95) and its ligand (CD95L) expression, and the percentage of apoptotic T cells assessed with Annexin V staining, at basal level and following activation.

The analysis will compare reproductive and immune function measures between the Oahu-born group and the two comparison groups controlling for relevant confounders. Secondary comparisons among the Oahu-born population will be made based on individual estimates of gestational heptachlor epoxide exposure during the milk contamination episode.

## Endocrine-Disrupting Pesticides and Neurodevelopmental Outcomes in Farmworker Children, Salinas Valley

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The primary goal of this study is to determine whether *in utero* exposure to endocrine-disrupting (ED) pesticides is associated with adverse effects on the neurobehavioral development of offspring of exposed women. The objectives are to: (1) determine whether *in utero* exposure to ED pesticides, such as organochlorine and nonpersistent ED pesticides, is associated with adverse effects on the neurobehavioral development of children; and (2) identify population correlates of exposure. To achieve Objective 1, this research project will determine whether levels of organochlorine pesticides in serum of women collected at 26 weeks gestational age or levels of nonpersistent ED pesticides measured in maternal urine collected prenatally (at 13 and 26 weeks gestational age, respectively) are associated with poorer performance on the Brazelton neurodevelopmental assessment. At older ages (6, 12, and 24 months postnatal), whether children's exposure to pesticides during the prenatal period is associated with poorer cognition, perception, attention, memory, motor coordination, and behavioral/emotional adjustment will be assessed using age-appropriate tests standardized and administered in Spanish. To achieve Objective 2, how levels of ED exposure vary by gender and age as well as which variables best predict exposure (e.g., occupational status, season, nearby pesticide use, and length of time in the United States) will be determined.

The study population consists of women and children participating in the University of California, Berkeley, Center for Children's Environmental Health Research in the Salinas Valley, CA, examining organophosphate pesticide exposures to pregnant women and their children as well as their association with adverse neurodevelopmental and respiratory effects in the children. It includes 601 pregnant women who were less than 20 weeks gestation, Medi-Cal eligible, 18 years or older, and received prenatal care at two community clinics. Of these, 88 percent prefer to speak Spanish, 85 percent were born in Mexico, and 53 percent have lived for 5 years or less in the United States. Women born in Mexico have been found to have elevated levels of organochlo-

rine pesticides in breast milk compared to levels found in U.S.-born participants. Possible sources of pesticide exposure during pregnancy included farm fieldwork (26%), other agricultural work (16%), other agricultural workers in the household (75%), pesticides used in the home (40%), and home within 200 feet of a field (15%). This research demonstrated that the study population has potentially heavy exposure to ED pesticides in the Salinas Valley, and has probable previous exposure to persistent ED pesticides in Mexico.

During Year 1, development was begun on pesticide use indices based on state pesticide use data, a unique resource in California. The association of these indices with exposure biomarkers will be evaluated. For each participant, the amount of nearby pesticide use is being computed for the 1- and 2-month periods prior to urine or serum sample collection. For the year 2000, approximately 600,000 pounds of potential ED pesticides were applied in the Salinas Valley. The Centers for Disease Control and Prevention (CDC) has developed assays for as many of the nonpersistent pesticides as possible. Currently, approximately 65 percent of these can be detected by urine, about 9 percent are detectable in serum only, and about 26 percent cannot be detected in urine or serum. All maternal urine specimens have been shipped to the CDC laboratory. Chemical analyses of the maternal urine samples began in Year 1 and will continue in Year 2. The CDC laboratory also will initiate measurement of biomarkers of exposure to 14 persistent organochlorine pesticides and polychlorinated biphenyls in archived serum samples collected at 26 weeks gestational age during Year 2. An extensive literature review was completed to identify mechanisms of ED for the ED pesticides. Neurobehavioral assessments have been completed on newborns and 6- and 12-month olds; and assessment of 24-month olds will continue until March 2003. Medical record abstraction for pregnant women through delivery is complete. Forms for pediatric medical record abstraction have been developed, and abstraction has been initiated for 24-month-old children.

## Persistent Organic Pollutants and Endometriosis Risk

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Endometriosis, a disease affecting approximately 5 percent of U.S. reproductive-aged women with chronic pelvic pain, dysmenorrhea, and infertility, has been linked in epidemiologic studies to exposures indicating high circulating estrogen levels. There has been recent public and scientific concern that endocrine-disrupting chemicals in the environment may have estrogenic effects in the body and therefore increase endometriosis risk, but results of the few epidemiologic studies of this issue have been mixed.

In this study, the relationship between endometriosis and exposure to organochlorine compounds and polychlorinated biphenyls is being examined in a large population-based study. Whether these associations are modified by polymorphisms in genes involved in estrogen metabolism will be determined. The study is an ancillary investigation to a current National Institute of Child Health and Human Development-funded case-control study of risk factors for endometriosis that is being conducted within a large health maintenance organization in western Washington State.

The parent study provides data from in-person interviews including reproductive, contraceptive, menstrual, and behavioral characteristics; dietary questionnaires; anthropometric measurements; pharmacy information; and results of laboratory analyses of serum for two polymorphic genes (GSTM1 and COMT) involved in detoxification and estrogen metabolism. The current study will utilize blood samples from 300 cases and 600 controls from the parent

study to determine serum levels of total polychlorinated biphenyls (PCBs), PCB congeners, hexachlorobenzene,  $\beta$ -hexachlorocyclohexane ( $\beta$ -HCH),  $\lambda$ -HCH, aldrin, heptachlor epoxide, oxychlorane, trans-nonachlor, p,p'-dichlorodiphenyl dichloroethylene (p,p'-DDE), o,p'-DDE, dieldrin, endrin, o,p'-dichlorodiphenyl trichloroethane (o,p'-DDT), p,p'-DDT, and mirex residues as well as two polymorphic cytochrome p450 genes (1A1 and 1A2). Additionally, 450 urine samples (150 cases and 300 controls) will be tested for levels of the methoxychlor metabolite 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE).

The primary objectives of the study are to determine: (1) whether the risk of endometriosis is associated with lipid-adjusted serum levels of the above-listed organochlorine pesticides or urine levels of HPTE; (2) whether the risk of endometriosis is associated with lipid-adjusted serum levels of PCBs (total PCBs and 35 PCB congeners); and (3) whether the risk of endometriosis resulting from organochlorine pesticide or PCB exposure differs among women with differing CYP1A1, CYP1A2, COMT, and GSTM1 genotypes. The secondary objective is to determine whether the risk of endometriosis resulting from organochlorine pesticide or PCB exposure differs among women with differing levels of other exposures affecting estrogen levels. Analyses comparing cases and controls with respect to levels of these organic pollutants, other hormonal risk factors, and their interactions with genetic polymorphisms will be conducted to address the specific objectives.



## Biogeographic Variation in the Model Organism *Palaemonetes pugio*: Implications for Toxicological Studies

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Toxicologists often have used “model” marine organisms such as grass shrimp (*Palaemonetes pugio*) to investigate the effects of potential toxins within estuarine environments. The species is widely distributed along the Atlantic Coast and readily available, making it a commonly used “sentinel” species target for toxicological studies. Though well studied in the laboratory, little is known about the “phylogeography” of the species, or how geography and species attributes interact to mold patterns of intraspecific genetic variation. If species are highly structured geographically, for example in *P. pugio*, then conclusions—and potentially management decisions—drawn from studies in one geographic locale might not be applicable to other areas. That is, it might be best if toxicological studies are constrained to comparisons involving areas with similar phylogeographic histories. For example, median lethal concentration (LC<sub>50</sub>) studies for the pesticide endosulfan from either side of the Florida Peninsula have produced toxicological data that are incongruent: susceptibility of shrimp from the Gulf of Mexico is reported to be one-half that of shrimp caught along coastal South Carolina (LC<sub>50</sub> = 1.31 µg/µL and 0.66 µg/µL, respectively). However, without knowledge of the background genetic variation between shrimp populations, a complete understanding of these data are difficult. The phylogeographic history of *P. pugio* throughout most of its range has been determined by examining mitochondrial DNA sequence variation in 161 individuals representing 21 populations. Phylogenetic

analyses showed a strong phylogenetic split between populations within the Gulf of Mexico and those along the Atlantic Coast. Interestingly, there was almost no divergence within the Atlantic Coast populations. In contrast, the Gulf of Mexico populations exhibited fixed genetic differences and strong population-level differentiation, suggesting that differences in life histories between populations in either basin are highly divergent or that they have widely divergent biogeographic histories. These shrimp currently are being analyzed at the nuclear DNA locus vitellogenin to test for congruence with the mitochondrial data that would suggest a longstanding divergence between the two regions. In either case, it is clear that individuals collected from these two genetically distinct regions might respond very differently to the same toxin. Thus, region-specific toxicological assessments appear to be more appropriate than species-wide generalizations.

These data have allowed for the design of a common garden experiment to test for susceptibility differences between regions. Because the two regions do not share haplotypes, sequence data can serve as an identifying marker. Shrimp from the Gulf of Mexico and the Atlantic Coast now can be placed into a common tank for toxicology testing, and identified as to origin afterwards by sequence data; thus eliminating questions surrounding protocol and methodology differences between different LC<sub>50</sub> studies.

## Persistent Organochlorine Compounds, Genetic Susceptibility, and Testicular Cancer Risk

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The incidence of testicular germ cell carcinoma (TGCC), the most common malignancy developing in young men, has increased several-fold since the 1950s. Experimental and observational studies in animal systems have raised concern that the increasing rates are due in part to population-wide, persistent exposure to endocrine-disrupting compounds from industrial and agricultural applications. Whether human exposure to such chemicals is associated with TGCC risk has not been directly studied.

This research project will determine whether the risk of TGCC is related to serum levels of persistent organochlorines, focusing on *p,p'*-1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p'*-DDE), polychlorinated biphenyls (PCBs), and other compounds (e.g., dieldrin, hexachlorocyclohexanes, hexachlorobenzene). The study also will examine whether the risk of TGCC associated with these compounds is modified by genetic susceptibility to mechanisms through which these compounds may alter TGCC risk. Specifically, it will determine whether TGCC risk is related to interactions between: (1) elevated serum *p,p'*-DDE and polyglutamine repeat tract polymorphisms in the androgen receptor (AR) gene; and (2) elevated serum PCB levels and polymorphisms in oxidative stress defense enzyme genes (e.g., glutathione *S*-transferases and superoxide dismutases).

An ancillary investigation to the Adult Testicular Cancer Lifestyle and Blood Specimen (ATLAS) Study—a National Cancer Institute-funded, population-based case-control study of molecular genetic risk factors for TGCC—will be conducted. ATLAS funding includes population-based case and control ascertainment and recruitment; a detailed in-person interview; blood collection; and molecular genetic analyses of polymorphisms in androgen synthesis, metabolism, and signaling genes (including AR). The ancillary study will include approximately 250 cases of TGCC and 750 controls recruited as part of ATLAS. Serum samples from cases and controls will be assayed for organochlorine pesticides and PCBs by high-resolution gas chromatography/isotope dilution high-resolution mass spectrometry, and genotyping for common polymorphisms in genes involved in oxidative stress defense systems (manganese superoxide dismutase, glutathione *S*-transferases A1, M1, P1, and T1) will be performed. Data on the AR and other androgen pathway polymorphisms from the main ATLAS Study will be incorporated. There will be adequate statistical power to detect relatively weak overall associations, as well as less than threefold interaction effects between organochlorine compounds and genetic polymorphisms. The results should add significant new information to the understanding of the role, if any, of environmental contaminants in the pathogenesis of TGCC.

## A Case-Cohort Study of Cryptorchidism, Hypospadias, and Delayed Sexual Maturation in a Dioxin-Contaminated Region: Chapaevsk, Russia

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Chapaevsk, Russia, with a population of 83,000, is located 43 km southwest of Samara on the Chapaevka River, which flows into the Volga River. One-half of the city is occupied by industries that are mostly of the military-industrial complex. One of the largest environmental polluters in Chapaevsk is the Middle Volga Chemical (SVZH, Himprom) Plant, which produced hexachlorocyclohexane (lindane) and its derivatives from 1967 to 1987. Since then, the plant has produced agricultural pesticides containing chlorine. By-products of the manufacturing process include dioxins and furans, which contaminated the region.

Cryptorchidism and hypospadias are common minor congenital anomalies of male reproductive tract development. Although several risk factors have been identified, the etiology of both disorders remains largely unknown. Recently, the possibility that these minor reproductive tract abnormalities may be related to exposure to environmental contaminants, such as the hormonally active dioxins, has been raised. Therefore, a study was designed to investigate the potential association of dioxin exposure to cryptorchidism, hypospadias, and delayed sexual maturation in adolescent-aged boys living in Chapaevsk.

The first phase of the study, conducted from March to May 1999, consisted of a survey of 10 to 16-year-old boys in Chapaevsk. Participants who agreed to take part in the study underwent a physical examination, with particular attention to the presence of cryptorchidism and hypospadias as well as the pubertal stage of maturation. Among 3,041 age-eligible boys identified via birth and school records, 2,580 (84.8%) boys were enrolled. Physical examinations of the children were performed by a pediatric endocrinologist and a urologist. Pubertal maturation was graded according to Tanner Staging for Genitalia, and pubic hair with testicular volume was determined

using an orchidometer (Prader beads). Testicular location and the presence of typical orchidopexy postoperative scars were noted. The presence of hypospadias was based on the location of the external urethral meatus. The children's birth records were reviewed to identify additional cases of cryptorchidism and hypospadias. The second phase of the study, conducted from October 1999 through May 2000, focused on a subset of 112 boys (14 to 16 years old) from Phase 1 of the study who were identified by the presence or absence of cryptorchidism, hypospadias, and delayed puberty, along with 134 controls chosen using a case-cohort design. These 246 children were targeted for a more thorough assessment, including blood and urine samples for organochlorine contaminant and hormone measurements, and administration of a detailed questionnaire on medical history, diet, and lifestyle. Among the 246 children, 221 (90%) mother-child pairs agreed to participate, and 208 (85%) of these completed the questionnaire. Blood samples collected from 200 mothers (81%) and 220 children (89%) were archived for later determination of dioxins, furans, and polychlorinated biphenyls.

The analysis presented in the poster will be a description of the study population with emphasis on residential proximity to Himprom and other potential predictors for dioxin exposure, which includes the residential address of the mother during pregnancy with index son, the work history of both parents prior to and during the index pregnancy, and children's consumption of foods that were locally grown (vegetables) or raised (cows, chickens, pigs). Whether these exposure surrogates are associated with case-control status for cryptorchidism, hypospadias, and/or delayed sexual maturation is being explored. Maps of the distribution of participants' residence location relative to the Himprom factory will be generated using an electronic map of Chapaevsk, constructed with the use of a geographic map of scale 1:10,000 and ArcView GIS 3.0.

## Study for Future Families II: Phthalates in Pregnant Women and Children

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The overall objective of this study, Study for Future Families II (SFFII): Phthalates in Pregnant Women and Children, is to assess the extent to which current levels of phthalates in the environment pose a risk to human reproductive health. To date, there have been no studies of the effects of *in utero* phthalate exposure on human development. The Centers for Disease Control and Prevention (CDC) recently reported that levels of monobutyl phthalate (MBP), a urinary metabolite of dibutyl phthalate, a reproductive animal toxicant, were significantly elevated in young women in a sample of the U.S. population. This research group conducted a pilot study of 52 pregnant women from Missouri and observed measurable levels of the four most prevalent phthalates in all subjects, although levels of MBP were somewhat lower than those seen in the CDC sample.

The Study for Future Families (SFFI): U.S. Study of Semen Quality in Partners of Pregnant Women, conducted by this research team in four U.S. cities, recruited a total of 800 pregnant women and their partners by July 2002, to compare time to pregnancy and semen quality among cities. Most subjects agreed to be recontacted regarding participation in followup studies, and the majority of women provided a urine sample while pregnant. SFFI mothers who agree to participate and their children constitute the study population for SFFII. Study centers for SFFII include three of the four SFFI Centers (Columbia, MO; Los Angeles, CA; and Minneapolis, MN) as well as a new center in Iowa City, IA. Pediatric physicians conduct standardized examinations on each child, including digital photographs of the breast and genitalia. Buccal smears and urine are being obtained on all children, and urine samples collected when infants are less than 6 months of age will be assayed for follicle-stimulating hormone (FSH).

Because the cost of phthalate screening all mother-child pairs is prohibitive, 50 boys and 50 girls will be selected as "atypical" if either: (1) they demonstrate definite

or probable genital or breast anomalies, or (2) measurements of their breast and/or genitalia fall in the extremes of the distributions for these parameters (e.g., for males, small penile size, excess pubic or breast tissue, decreased anogenital distance (AGD); for females, small clitoral ratio, decreased AGD, excess breast or pubic fat). These children will constitute the "case" group. Each case will be matched (1:1) on sex, gestational age, center, and ethnicity to a child for whom all measurements fall within normal limits.

These 200 mother-child pairs will comprise the study population for a nested case-control study. Urinary phthalate metabolite levels will be measured in prenatal and postnatal maternal samples as well as in samples collected during the child's first and second years of life. Phthalate metabolite levels in the mother's prenatal urine will be examined in relation to outcomes from the pediatric examination, including genital parameters, amount of breast tissue, and FSH level. These investigators will seek to identify sources of phthalate exposure by relating mothers' self-reported use of phthalate-containing products (e.g., soaps, cosmetics, teething rings, nipples, and other plastics) at the time of urine collection to measured phthalate metabolite levels. A sample of breast milk, or the infant's usual formula, also will be collected at the first visit and stored for future analyses, together with the previously stored maternal serum and pre- and postnatal urine samples.

The award was received in January 2002, and much of the first study year was spent developing study documents and protocols, which will be summarized in this presentation (and available on the SFFII Web Site). Pediatric physicians and nurse assistants were centrally trained to perform the specialized genital examination using a training video developed for this purpose by study researchers. Recruitment began at most centers in May 2002, and progress as of October 1, 2002, will be presented.

## Laboratory and Mesocosm Measurements of the Stereoselective Degradation of Endosulfan

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The pesticide endosulfan is known to biodegrade in the environment to the stable and bioaccumulative endosulfan sulfate. In the laboratory, these researchers observed that the stereoisomer hydrolyzed more rapidly than the form. The rate of hydrolysis was measured in the laboratory in solution and in a variety of suspensions, in simulated sunlight, and in the dark. Suspended solids included sea sand,  $\text{TiO}_2$ ,  $\text{Fe}_2\text{O}_3$ ,  $\alpha\text{-FeOOH}$ , Laponite<sup>®</sup>, and  $\text{SiO}_2$ . In all cases, there was no effective photochemical processing, but there was a clear selectivity for the hydrolysis of  $\beta$  endosulfan over  $\alpha$ . This observation was explained by a more stable transition state for  $\beta$ -endosulfan that was confirmed with *ab initio* molecular orbital calculations (STO-6G) on the anionic intermediates of endosulfan hydrolysis. The first hydrolysis product was endosulfan diol; a series of hydrolytic and oxi-

dation processes (mechanism unknown) yielded a terminal product, endosulfan hydroxy acid. The chemical characteristics of this new degradation product are reported. The effect of stereoselective hydrolysis on endosulfan in the environment was measured in three model estuarine mesocosms (aqueous volume approximately 300 L, *Spartina*, and associated sediment dwellers) that were spiked with  $\alpha$ ,  $\beta$ , or a mixture of  $\alpha$  and  $\beta$  (technical grade). It was observed that  $\beta$  endosulfan hydrolyzed rapidly, so comparatively little was available for biodegradation and conversion to sulfate. From these measurements, it was determined that approximately 75 percent of total endosulfan-to-endosulfan sulfate conversion in estuaries is from biodegradation of the  $\alpha$  stereoisomer. Future work will focus on the fate of the hydroxy acid.

## Multivariate Modeling of the Photolysis of Aqueous Fipronil

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In this study, the correlation between laboratory and mesocosm studies of the fate of the phenylpyrazole insecticide fipronil is reported. A multivariate model of the fate of fipronil was developed in the laboratory to predict its degradation rate and product profile in the presence of sunlight, natural organic matter, bicarbonate, and nitrate. There were several abiotic pathways available for fipronil degradation in this system, including direct photolysis and oxidation by hydroxyl radical, singlet oxygen, or hydrogen peroxide. However, product studies indicated that fipronil was quantitatively converted to desthio-fipronil, a product that is associated with direct photolysis alone. It was observed that natural organic matter acted to decrease the rate of fipronil degradation, either by competition for photons or transient oxidants. This model was applied to predict the fate of fipronil in a series of modular estuarine mesocosms at the National Ocean Service's Center for

Coastal Environmental Health and Biomolecular Research in Charleston, SC. In these experiments, the loss of aqueous fipronil (single-dose experiment, with initial fipronil concentrations of 355 and 5,000 ng/L, three replicates at each concentration) was monitored over 28 days. Although the direct photolysis product was detected, the mass balance was dominated by fipronil-sulfone and fipronil-sulfide, products that are a signature of fipronil biodegradation.

Nonetheless, direct photolysis appeared to account for 3-14 percent of all fipronil loss in the model environments, at 10 percent ambient ultraviolet-B (limited by the structure of the mesocosm). All products were confirmed by comparison of retention times (gas chromatography-electron capture detection) and mass spectra (gas chromatography-ion trap mass spectrometry) to synthesized standards.

## Exposure to DDT and DDE in Relation to Menstrual Cycle Length Among Laotian Immigrants

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This study was initiated because of concern about the effects of endocrine-disrupting chemicals (EDCs) on reproductive capacity in wildlife and laboratory animals, and the fact that there are few data available in humans. It was designed to examine the effects of potential EDCs on ovarian function, as measured by the frequency of menstrual cycle characteristics, in Laotian immigrants who may have higher exposure from their home country as well as from sport fish consumption. Working with local Lao communities, Lao field workers were hired and trained to recruit women of reproductive age (18-39) who were born in Southeast Asia and regularly consumed fish. Participants were asked to complete two interviews, provide a blood sample, and collect first morning urine samples daily during three menstrual cycles. The urine samples were assayed for metabolites of estrogen and progesterone, from which several menstrual cycle parameters were defined. These methods were similar to those of a larger study conducted among a general population. The serum was assayed for organochlorine compounds including pesticides, polychlorinated biphenyls, and polybrominated diphenyl ethers.

The target of 50 women completed the study. Urine was collected during 148 complete cycles, with information on an additional 39 cycles that had partial urine collection. The mean cycle length in complete cycles was 30.4 days (standard deviation [SD] 7.0). Focusing on dichlorodiphenyltrichloroethane (DDT) and its metabolite 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE), these investigators found that all women had detectable levels, with a mean of 1.7 ppb (SD 3.5) and

20.3 ppb (SD 22.5) respectively, indicating higher body burdens than currently found in comparable U.S. populations. The covariates related to mean exposure level included age, parity, breastfeeding, and time spent in Thailand, some of which were inter-related. Examining quartiles of exposure, cycle length was decreased with increasing exposure in preliminary analyses. Using methods that account for repeated measures, at the highest quartile of DDE exposure mean cycle length was decreased by 3.5 days (95% confidence interval [CI] -7.6, 0.58) and at the highest quartile of DDT exposure by 4.4 days (CI -8.2, -0.58), compared to the lowest quartile. Adjusting for lipid level attenuated the decrements only slightly. Adjusting for other demographic variables attenuated the decrements in cycle length for DDT, but not DDE. Examining the log of DDE or DDT as a continuous variable, decreasing cycle length with increasing exposure was found that was statistically significant for DDE. There was a decrement in luteal phase length examining either the quartiles or logged body burdens.

In summary, these data suggest a possible association of DDE/DDT exposure with changes in menstrual function, but the findings are based on small numbers and potentially confounded by other exposures. If exposure is associated with shorter cycles, this could reflect a hormonal pathway. Some laboratory studies have indicated that DDE may interfere with progesterone, which would be consistent with an effect during the luteal phase, when progesterone is primarily secreted. The investigators plan to examine hormone endpoints and the other EDC exposures as well.

## **Mechanisms of Action**



## Alteration of Calcium-Dependent Cell Signaling as a Potential Mechanism of Endocrine Disruption

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The importance of calcium-dependent signal transduction in mediating hormonal control of ovarian sex steroid production has been demonstrated in numerous vertebrate species, including the Atlantic croaker (*Micropogonias undulatus*). Additionally, studies have shown that specific components of calcium-dependent signal transduction pathways, including calcium channels and calmodulin, are targets for environmental chemicals such as the heavy metals cadmium, lead, and mercury as well as the pesticides lindane, 4-octylphenol, and o,p'-dichlorodiphenyltrichloroethane (o,p'-DDT). However, there is little evidence directly linking these actions of xenobiotic chemicals to adverse endocrine effects, such as impairment of steroidogenesis. Therefore, the goal of this research project is to investigate environmental chemical disruption of calcium-dependent signaling as a potential novel mechanism of endocrine disruption in the Atlantic croaker.

**Primary cell culture.** A primary cell co-culture system for ovarian theca and granulosa (T-G) cells was established to conduct specific experiments investigating the direct actions of environmental chemicals on calcium-signaling pathways. T-G cells were obtained by enzymatic digestion of mature ovarian tissue and isolated from other cell types by density-gradient centrifugation. These cells adapted well to culture conditions and produced high levels of testosterone in response to gonadotropin for at least 5 days. Croaker T-G cells maintain their endocrine function in primary culture, and thus are a useful model for investigating mechanisms of endocrine disruption.

**Signal transduction pathways.** Previous research on croaker whole ovarian follicles has demonstrated a clear role for calcium-dependent cell signaling in modulating ovarian steroidogenesis. Therefore, experiments were conducted on T-G cells to determine if these pathways are maintained in culture. Calcium ionophore

A23187 (0.25  $\mu$ M), a drug that increases cytosolic calcium concentrations, stimulated basal testosterone production twofold. Gonadotropin-induced testosterone production was attenuated by the voltage-sensitive calcium channel (VSCC) blocker verapamil (1-10  $\mu$ M) and the calmodulin inhibitor W-7 (20  $\mu$ M). These data show that increases in cytosolic calcium concentrations via ion transport through VSCCs and activation of calmodulin are required to transduce the initial hormone signal and stimulate steroid production.

**Effects of environmental chemicals on steroidogenesis.** Because the chemicals cadmium and o,p'-DDT have been shown to alter calcium function, preliminary experiments were conducted to examine their effects on the endocrine function of T-G cells. Cadmium (10 ppm) significantly impaired hormone-induced testosterone production. Alternatively, treatment with low doses of o,p'-DDT (0.001-0.01 ppm) stimulated basal steroidogenesis. These results demonstrate that a viable and sensitive bioassay has been established for investigating effects of environmental chemicals.

**Effects of xenobiotics on cytosolic calcium.** Experiments are ongoing to determine if these chemicals directly modify calcium-signaling pathways. Epi-fluorescence, time-lapse microscopy will be used to measure both spatial and temporal cytosolic calcium changes in individual T-G cells exposed to heavy metals, pesticides, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and other chemicals to determine if they directly alter intracellular calcium concentrations under basal or hormone-stimulated conditions. Due to the ubiquitous nature of calcium signaling among cell types and organisms, the results of this study will be applicable to a wide variety of calcium-dependent processes in many different tissue types and species. Therefore, this research has the potential to significantly contribute to the general understanding of endocrine disruption.

## Elevations of Estradiol in the Cycling and Ovariectomized, Estradiol-Implanted Female Rat by the Drinking Water Disinfection By-Product Dibromoacetic Acid

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The chlorination of municipal drinking water has been one of the most successful public health interventions to combat disease. However, a variety of treatment-related chemicals are generated in the process that have been suspected of having adverse effects in both humans and test animal species. Although some positive associations have been drawn between various disinfection by-products (DBPs) and some forms of cancer, there are indications that reproductive functions may be compromised as well. The haloacetic acids are one of the principal types of DBPs generated by chlorination, and two members of this class, dibromoacetic (DBA) and dichloroacetic acids, have been reported to affect sperm production and gonadal hormonal activity in the male rat. At high doses, DBA also has been found to induce a persistent alteration in cyclicity in the female Sprague-Dawley (S-D) rat, which has been confirmed in the present study using 14-day oral exposures (0-270 mg/kg). Body weights during dosing remained unaffected.

Preliminary results suggested that an alteration in estradiol (E2) concentrations may have contributed to this effect on cycling status. To investigate this possibility further, normal-cycling S-D female rats (60-90 days) were gavaged daily with DBA (0, 30, 90, or 270 mg/kg in water, pH adjusted to 6.8) for 2 weeks. After 8 days of treatment (during which cycles were maintained), all animals were ovariectomized (OVX), and 3 days thereafter implanted subdermally with silastic capsules (6 mm in length) containing estradiol benzoate (4 mg/mL in sesame oil). Blood was sampled by tail nick at 24, 48, and 72 hours postimplant. Capsules containing vehicle only also were implanted, and blood was sampled at 72 hours for 0 and

270 mg/kg-treated rats. Intact rats with regular 4-day cycles also were gavaged with DBA (0, 30, 60, or 120 mg/kg) for 2 weeks as above, and blood samples were taken during the last 4 days of treatment. Cycling status was monitored to ensure that normal cycles were maintained at these dosages. Only those females continuing to exhibit regular 4-day estrous cycles were retained. For OVX rats, data showed that by 72 hours, treatment with DBA caused a distinct dose-related elevation in E2, and that the highest dose had a mean concentration approximately 2½-fold above the controls. For intact, 4-day cycling rats, E2 concentrations remained elevated on the day of estrus for the two highest dose groups, suggesting that this effect may have contributed to the emerging disruption in cyclicity previously observed.

To explore further whether the elevation in E2 observed in the OVX/E2-implanted rats was due to an impairment in E2 metabolism, females were gavaged with DBA (0 or 270 mg/kg) for 2 weeks. During the final 3 days, they were given phenobarbital (PhB, sodium salt) either in the drinking water (0.1%) or by intraperitoneal injection (20 mg/kg) to increase hepatic E2 metabolism. Neither PhB route caused a lowering of the DBA-induced E2 elevations, suggesting that the effects were linked to an alteration in the clearance of the hormone or a suicide inhibition of hepatic P450 activity. The data also indicate that although DBA administered over the course of 2 weeks in adult rats can act as an endocrine-disrupting chemical at concentrations in excess of those levels permissible for human exposure, it is conceivable that lower dosages given over more extended treatment periods could have similar adverse effects on reproductive function.

## Testosterone:Fatty Acid Esterification: A Novel Target of Endocrine Disruption Caused by Tributyltin

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Imposex, the development of a penis and/or vas deferens in female gastropods, is regarded as the best example of endocrine disruption in an invertebrate linked to an environmental pollutant. This masculinization of females is causally associated with increased testosterone levels resulting from exposure to the marine biocide tributyltin (TBT). Endocrine disruption in gastropods is of global concern, with imposex having been demonstrated in more than 150 species worldwide. The development of screening approaches to detect this mode of endocrine disruption is contingent on elucidating the precise endocrine process that is affected by TBT. The objectives of this study were to: (1) elucidate major testosterone biotransformation processes in the mud snail (*Ilyanassa obsoleta*) that may be disrupted by TBT, resulting in increased testosterone levels; and (2) assess the effects of TBT on these processes. The study discovered that testosterone undergoes limited biotransformation in the mud snail to aromatized, hydroxylated, or oxidoreduced derivatives. Rather, testosterone is predominantly converted to apolar esters that are retained by the organisms. These esters are largely fatty acid conjugates that are produced by one or more acyl CoA:testosterone acyltransferase enzymes. It was hypothesized that fatty acid esterification of testosterone serves to store excess testosterone in a lipid-soluble form that could be drawn on as testosterone is needed by the organism. To test this hypothesis, snails were treated with various regimens of 4-nonylphenol or testosterone to decrease or increase, respectively, total testosterone levels. Total, free (unesterified), and esterified testosterone levels then were evaluated in individual snails. The amount of es-

terified testosterone present in these organisms increased in direct proportion to the level of total testosterone, while free testosterone levels remained relatively constant. Therefore, in this species, testosterone esterification maintains free testosterone by removing and storing testosterone when levels are in excess, and perhaps by providing testosterone when levels are below normal.

The effects of TBT on this major testosterone homeostatic process next were investigated. Snails were exposed to environmentally relevant concentrations of TBT, and free, esterified, and total testosterone levels were assessed. Total testosterone levels in snails were not altered by TBT; however, free testosterone levels increased with increasing TBT exposure concentration. Further, the retention of testosterone fatty acid esters was decreased with increasing exposure concentration of TBT. These results indicate that TBT elevates free testosterone levels in snails by decreasing the accumulation of fatty acid esters. A comparative assessment of testosterone homeostasis in snails collected from a tin-impacted and non-impacted site confirmed that tin-exposed, imposexed snails retain less testosterone:fatty acid ester and more free testosterone.

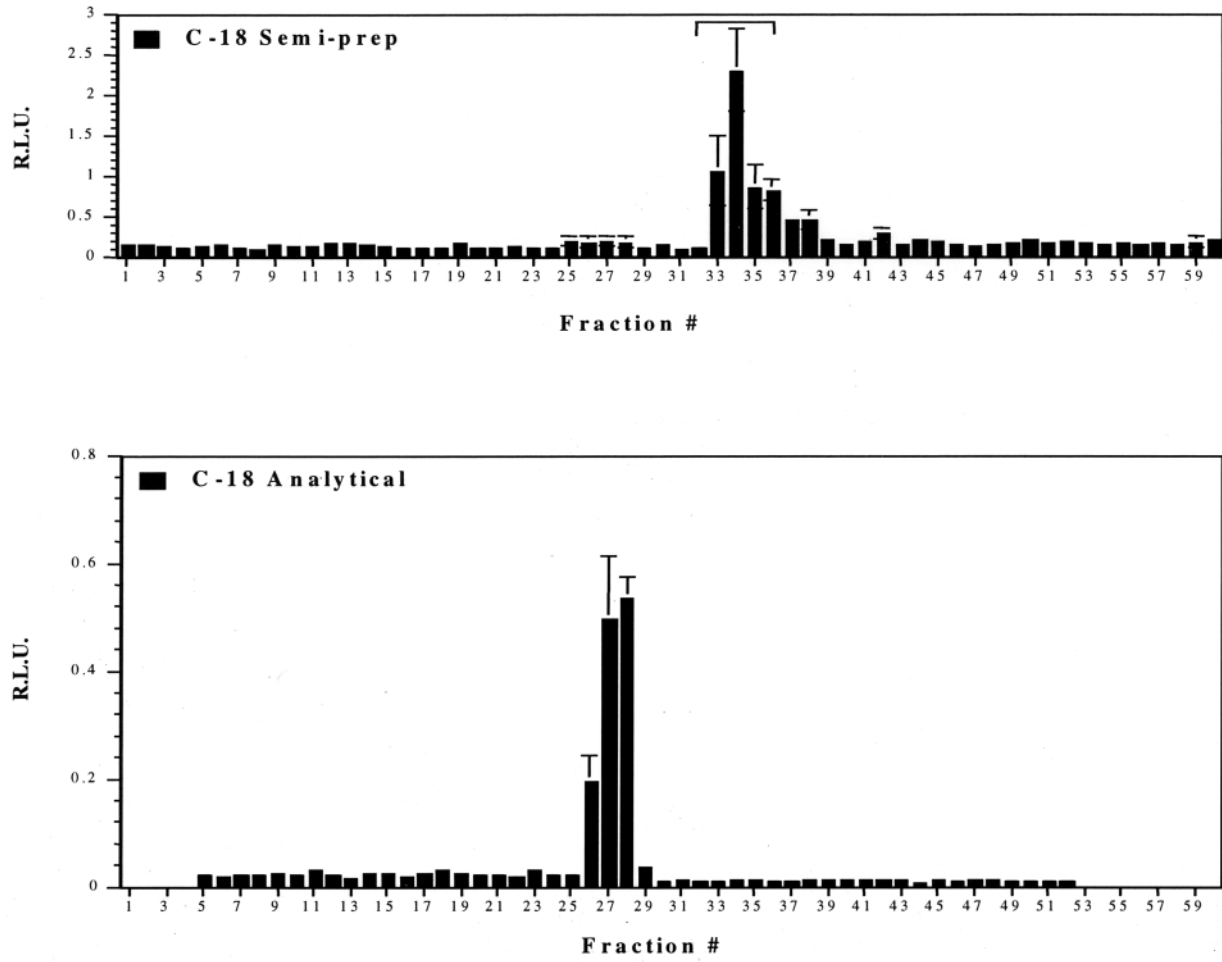
Results from this study have identified a novel target of endocrine disruption—the fatty acid esterification of testosterone, which may be responsible for TBT-induced imposex in snails and also may be of relevance to mammals.

## **HPLC Purification of an Endocrine-Disrupting Retinoid Activity From a Minnesota Lake With a High Incidence of Malformed Amphibians**

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There is considerable evidence that wildlife and domestic animals have suffered adverse consequences from exposure to environmental chemicals that interact with components of the endocrine system. To assess the significance of endocrine disruptors that activate retinoid-signaling pathways for their role in causing limb developmental deformities in frogs, and to understand their mechanism of action to assess their implications for human health, this research project studied the effects of disruption of retinoid-sensitive signaling pathways. These studies demonstrated that all the malformations observed in wild populations of frogs can be induced by experimental exposure to retinoids. The model that environmental retinoids is the cause of frog deformities

predicts that retinoids will be found at sites where deformed frogs are found. To identify such environmental retinoids, hydrophobic substances are extracted from water samples using solid phase extraction. These substances then are fractionated by high-performance liquid chromatography and tested for their ability to activate the retinoic acid receptor in transient transfection assays (increased Relative Luciferase Units; see Figure 1). Active fractions are purified to homogeneity as judged by ultraviolet absorption spectra and then analyzed by electrospray and electron impact mass spectroscopy for exact mass determination. Mass spectra will be used to derive the compound's molecular formula and molecular structure.



**Figure 1.** Retinoic acid receptor activation by high-performance liquid chromatography fractions of water from a lake in Minnesota (R.L.U. = Relative Luciferase Units).

## Differential Binding of Estrogens and Estrogenic Compounds to ERs Alpha, Beta, and Gamma of the Atlantic Croaker, *Micropogonias undulatus*

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These researchers recently described a novel form of estrogen receptor (ER), ER gamma, in a teleost fish, the Atlantic croaker. Atlantic croaker ER gamma (acER gamma) is genetically distinct and has a different tissue distribution pattern than acER alpha or beta. Phylogenetic analysis shows that ER gamma is present in other teleost fish, but was not recognized as such due to lack of evidence for three ERs in these species. ER gamma shares a high degree of amino acid similarity with other ERs. However, acER gamma differs from acER alpha and acER beta in the ligand-binding domain (LBD), and these differences are conserved in all other ER gammas. To determine whether these amino acid changes have led to differences in ligand-binding specificity between the three ER types, ER fusion proteins were created for bacterial expression including the E and F domains of acER alpha, acER beta, and acER gamma. Bacterially expressed fusion proteins for acER alpha, beta, and gamma show specific, high-affinity binding to [<sup>3</sup>H] estradiol (E2) with dissociation constants of  $0.61 \pm 0.013$ ,  $0.40 \pm 0.006$ , and  $0.38 \pm 0.059$  nM, respectively. The rank orders of bind-

ing to the acER fusion proteins are DES >> ICI182 > TOH > ICI164 > E2  $\geq$  ZEAR > MOX E > TAM > E1  $\geq$  17 $\beta$ E2 > E3 > 2OH E = GEN >> RU 486 for acER alpha; ICI182 > DES > TOH > E2 > ICI164 > GEN > MOX E > TAM > ZEAR = E1 > E3 = 17 $\beta$ E2 > 2OH E >> RU 486 for acER beta; and E2  $\geq$  DES > TOH > ICI182 > ICI164 > E3  $\geq$  GEN > MOX E > ZEAR > E1 > 17 $\beta$ E2 > RU 486  $\geq$  TAM > 2OH E for acER gamma.

The differences in relative binding affinities that acER alpha, beta, and gamma fusion proteins show for native estrogens and other estrogenic compounds suggest that amino acid changes in the LBD may confer different functional properties to the three ER subtypes. These researchers currently are investigating the binding characteristics of xenoestrogens such as kepone, dichlorodiphenyl-trichloroethane, and polychlorinated biphenyls to the three ER subtypes. Differential binding of xenoestrogens to ER subtypes could result in distinct physiological responses to environmental exposures, depending upon the ER subtype population makeup of target organs.

## A Short-Term Dosing Model for Detecting the Effects of Environmental Contaminants on Thyroid Hormones in the Rat: Effects of Pesticides

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Recently, a short-term rat-dosing model was developed to examine the effects of environmental mixtures on thyroid homeostasis (TH). Prototypic chemicals such as dioxins, polychlorinated biphenyls, and polybrominated diphenyl ethers have been tested and shown to adversely impact both neurological development and TH, primarily by upregulating catabolism of thyroid hormones by liver enzymes. Current efforts examined the effects of select pesticides in this model. Female Long Evans rats (28 days old) were orally dosed for 4 consecutive days with the dithiocarbamate fungicide mancozeb (MAN) or the herbicide pronamide (PRO) (0, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 250, 500, 1,000 mg/kg/day), or the dithiocarbamate fungicide thiram (THI) (0, 6.25, 12.5, 25, 50, 100, 200, 400, 800 mg/kg/day). Serum and liver samples were collected 24 hours after the last dose. Triiodothyronine (T3)

and thyroxine (T4) were measured via radioimmunoassays. Hepatic ethoxy-resorufin-O-deethylase (EROD), pentoxy-resorufin-O-deethylase (PROD), and uridinediphosphate-glucuronosyltransferase were determined in hepatic microsomal fractions. Liver-to-body weight ratios (LBR) increased for PRO at doses of  $\geq 125$  mg/kg/day, for MAN at  $\geq 500$  mg/kg/day, and for THI at  $\geq 400$  mg/kg/day. PRO, MAN, and THI all produced similar dose-related decreases in T4, with estimated median effective doses of approximately 250 mg/kg/day. Potencies for decreases in T3 were THI > PRO > MAN. Maximal suppression of T3 and T4 was approximately 50 percent and 80 percent at the highest doses, respectively. Neither MAN nor THI caused changes in EROD or PROD. These data suggest that this rodent-dosing model is sensitive to short-term perturbations in thyroid hormones caused by these pesticides.

## Impact of Chemical Mixtures on Steroid Metabolism in Mud Snails (*Ilyanassa obsoleta*)

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Imposex snails are found in harbors and near marinas where they often are exposed to a wide mixture of chemicals. The antifouling compound tributyltin (TBT) has been causally linked to the induction of imposex in gastropods; however, the effect of co-exposure to other environmental contaminants has not been studied. Although TBT is a cytochrome P450 inhibitor, many environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) induce the expression of cytochrome P450. Mud snails (*Ilyanassa obsoleta*) were dosed with TBT (20 ng/L) and/or the model PAH 3-methylcholanthrene (3-MC) (10 nM) for 45 days. The snails were examined for the induction of imposex and changes in P450 activity as well as for effects on steroids biosynthesis.

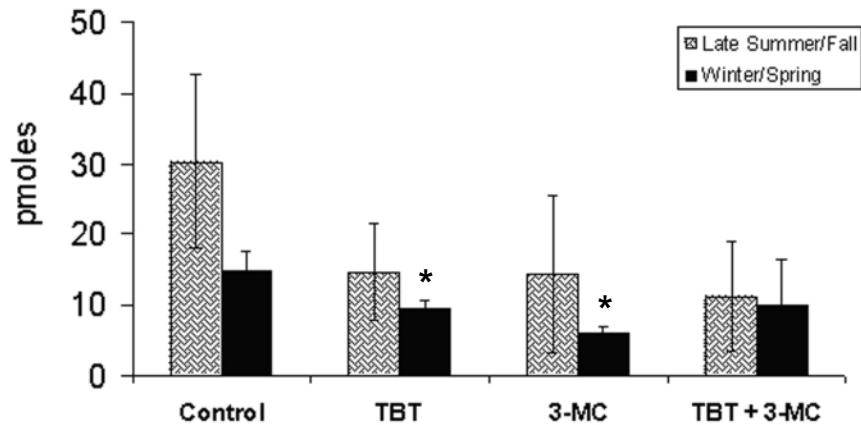
Control snails had an imposex level of 1.93 percent with a relative penis size index (RPSI) of 0.005, TBT-exposed snails exhibited a 40.42 percent imposex induction with an RPSI of 2.07, 3-MC snails exhibited a 5.05 percent imposex induction with an RPSI of 0.001, and TBT + 3-MC snails exhibited an 11.95 percent imposex induction with an RPSI of 0.880. Snails exposed to TBT exhibited a decrease in P4501A activity (7-ethoxycoumarin O-deethylation activity = 0.0003 nmol/min/mg compared to 0.0055 nmol/min/mg for controls). Snails exposed to 3-MC exhibited an increase in activity (0.0095

nmol/min/mg), while animals exposed to both TBT and 3-MC had an intermediate level of activity (0.0068 nmol/min/mg).

Analysis of steroid metabolism indicated that significant effects occurred *in vivo* in snails exposed to TBT or 3-MC. Androstenedione metabolism was measured by the <sup>3</sup>H<sub>2</sub>O-release assay. Snails exposed to TBT or 3-MC both exhibited a significant decrease (approximately 50%) in their conversion of androstenedione to testosterone. Co-exposure of snails to both contaminants resulted in metabolic activity similar to the controls (see Figure 1). In addition, other P450-mediated steroid pathways such as the metabolism of pregnenolone to 17 $\alpha$ -hydroxypregnenolone or progesterone to androstenedione were inhibited by exposure to TBT or 3-MC.

This clearly indicates that although TBT does inhibit P450 aromatase activity, this inhibition does not account for increases in testosterone in the exposed animals. 3-MC also inhibited aromatase activity, yet had no effect on imposex induction. In addition, it is quite evident that each of the P450-mediated steroid pathways was significantly affected by exposure to the contaminants. Therefore, it is reasonable to assume that a more diverse mechanism is responsible for imposex induction.





**Figure 1.** Aromatase activity in mud snails exposed to 20 ng/L TBT or 10 nM 3-MC. Concentration is given as the mean ( $n = 10$ )  $\pm$  standard deviation. Asterisks indicate a significant difference from the controls.

## Mechanisms of Toxicity of Dioxin-Like Compounds to Primate Ovarian Cells

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Endocrine disruptors such as 2,3,7,8-tetra-chloro-dibenzo-p-dioxin (TCDD) induce abortion in non-human primates and decrease estradiol (E2) production at the level of the ovaries. To better understand how this endocrine disruptor acts on its target tissue, the effects of TCDD exposures on steroidogenesis were studied in human luteinized granulosa cells (hLGC). TCDD inhibited the secretion of E2 without affecting either progesterone or 17 $\alpha$ -hydroxyprogesterone. It was hypothesized that the molecular target of TCDD, inhibiting E2 production by hLGC, was 17 $\alpha$ -hydroxylase/17,20-lyase cytochrome P450 (P450c17), the enzyme catalyzing the synthesis of androgens. In support of this hypothesis, TCDD (10 nM) decreased E2 production without altering either E2 metabolism or levels of aromatase cytochrome P450 (P450arom). The decrease in E2 induced by TCDD was ameliorated by the addition of androgen substrates, but not substrates earlier in the metabolic pathway. An anti-human P450c17 antisera was developed, and a direct radiometric assay of the 17,20-lyase activity of P450c17 was adapted to further test the hypothesis. Western immunoblot analysis demonstrated that TCDD treatment of hLGC decreased the expression of P450c17 by as much 50 percent. 17,20-lyase was more than 10-fold lower than P450arom activity and therefore likely limited E2 synthesis. TCDD induced a 65 percent decrease in 17,20-lyase activity, but no changes were seen in either P450arom or in nicotinamide adenine dinucleotide phosphate-cytochrome P450 oxidoreductase (reductase) that supports these microsomal P450 activities. This research study has concluded that the molecular target for endocrine disruption of hLGC by TCDD is P450c17, acting to specifically decrease the supply of androgens that are essential for E2 synthesis, and that

the toxic effect does not involve either P450arom or the redox partner protein reductase.

It is well known that TCDD has a long half-life *in vivo*. To test the hypothesis that this endocrine disruptor can have persistent adverse effects on female reproductive health, the ovarian function in mature female cynomolgus macaques was evaluated more than 1 year after a single exposure. Urinary estrone conjugates (E1C), pregnanediol-3-glucuronide (PdG), and follicle-stimulating hormone (FSH) were measured in animals following a single oral exposure (1, 2, or 4  $\mu$ g/kg BW) to TCDD. Three out of four animals in the high-dose group revealed no evidence of menstrual cycles. These noncycling animals had baseline E1C concentrations without ovulatory mid-cycle peaks and monotonic PdG profiles. Mean FSH concentrations during the mid-follicular phase of the medium dose group and during the entire cycle of the high-dose group were elevated, and the endometria of the noncycling animals were inactive. These data demonstrate that a single exposure of TCDD leads to long-term adverse effects on ovarian function in non-human primates, most likely by blocking estrogen production.

The next steps are to: (1) isolate RNA for a microarray analysis to identify the gene(s) that may be up- or down-regulated by the TCDD treatment on hLGC; (2) analyze the production of transcript for P450c17 by reverse transcriptase polymerase chain reaction; (3) examine the rate of transcription of P450c17 and reductase by nuclear runoff assay; and (4) investigate the effects of TCDD on phosphorylation of P450c17 and reductase by incubating with <sup>32</sup>P-phosphoric acid followed by immunoprecipitation with specific antisera.

## Developmental Toxicity of Antiecdysteroids in the Crustacean *Daphnia magna*

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Ecdysteroids, polyhydroxylated steroid hormones, regulate the molting processes in crustacean and other arthropod species. These researchers have shown that ecdysteroids also play an important role in crustacean embryo development. It was hypothesized that environmental chemicals eliciting antiecdysteroidal activity could be detrimental to crustacean embryo development and fecundity. Various chemicals were evaluated for potential antiecdysteroid activity. Fenarimol, an agriculture fungicide, and testosterone delayed molting of neonatal daphnids (*Daphnia magna*) in a concentration-dependent fashion, and this effect was mitigated by co-exposure to exogenous 20-hydroxyecdysone. These results implicated these compounds as having antiecdysteroid activity. Exposure of either gravid maternal organisms or isolated embryos to fenarimol or testosterone resulted in embryo abnormalities that could be prevented by co-exposure to 20-hydroxyecdysone. Abnormalities of fenarimol-exposed isolated embryos were associated with the late stages of development; while testosterone-exposed isolated embryos exhibited abnormalities associated with both early and late stages of development. Fenarimol, but not testosterone, was found to lower endogenous ecdysone levels. This effect presumably was due to the known ability of fenarimol to inhibit steroidogenic enzymes. Conversely, testosterone, but not fenarimol, was found to inhibit the action of ecdysteroids in an ecdysone-responsive cell line. These results suggest that testosterone exhibits

antiecdysteroid activity by acting as an ecdysone receptor antagonist.

Analysis of ecdysone levels in the normal developing embryo revealed that ecdysone levels initially are high and progressively decrease through mid-development. Ecdysone levels then increase during the latter stages of development. It was surmised that the initial pool of ecdysone in the embryo is of maternal origin, and the latter pool results from *de novo* synthesis in the embryo following organogenesis. Consistent with the demonstrated modes of actions of fenarimol and testosterone, the inhibitor of ecdysone synthesis (fenarimol) elicited effects on isolated embryos that were associated only with the pool of newly synthesized ecdysone. The ecdysone receptor antagonist (testosterone) elicited effects on isolated embryos that were independent of the ecdysone pool involved. Both fenarimol and testosterone were found to reduce fecundity of daphnids at exposure concentrations that elicited antiecdysteroid activity. These results demonstrate mechanisms of action and consequences of antiecdysteroids. Results also demonstrate the utility of isolated daphnid embryos to detect antiecdysteroid activity of chemicals and provide mechanistic information regarding the source of this activity. Future studies will focus on modeling and experimentally demonstrating the consequences of exposure to combinations of chemicals that elicit antiecdysteroidal activity through various mechanisms.

## Levels of the Neuropeptide Hormone APGWamide Are Elevated in TBT-Dosed Snails and in Snails Transferred to a Contaminated Site

Eva Oberdörster<sup>1</sup> and Patricia D. McClellan-Green<sup>2,3</sup>

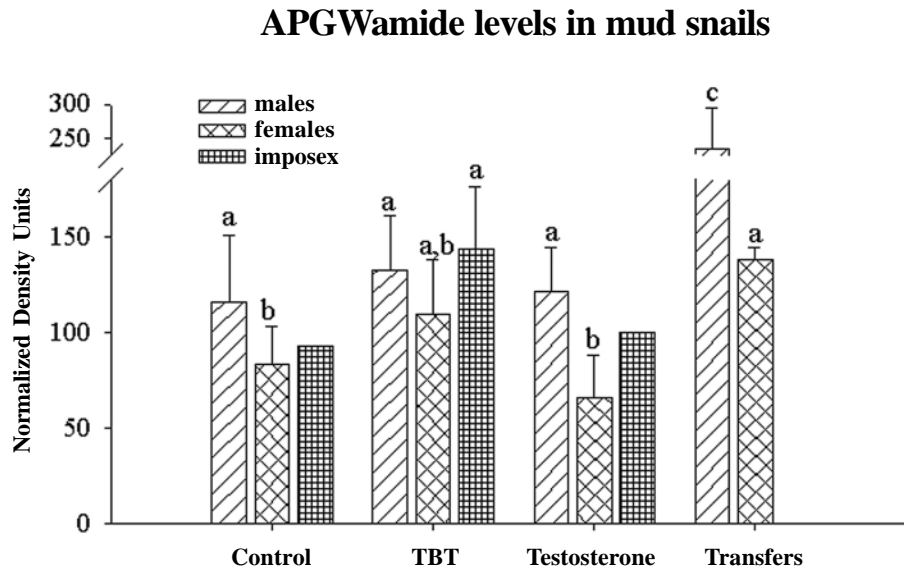
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APGWamide, the putative penis morphogenic factor that normally causes male sex characteristic development in breeding male snails, also can induce imposex in female snails at very low doses ( $10^{-16}$  moles) and in a short time period (7 days). In this study, snails were either collected from a relatively pristine estuarine reserve and injected subcutaneously with either 20 ng tributyltin (TBT), 500 ng testosterone, or vehicle (EtOH) controls. In addition, snails from the pristine location were caged for 3 months in a site that historically has 100 percent imposex females and males without regressing sex organs. APGWamide immunoreactivity was measured in snail body homogenates via Western blotting. Samples were run in duplicate, and an internal standard was run in all gels. Results are given as percent of the internal standard. Gels also were run with increasing levels of APGWamide, and standard curves were generated showing linearity over a wide range of APGWamide concentrations.

The results show that control males had significantly higher APGWamide levels than control females (see Figure 1). All TBT-treated animals, whether male, female,

or imposex, had levels of APGWamide similar to control males and significantly higher than control females. In testosterone-treated animals, APGWamide levels were identical to the corresponding vehicle controls. That is, males had similar levels to control males; females had similar levels to control females, and imposex animals ( $n = 1$ ) also were not different from one another (additional data analysis ongoing). This indicates that there may be two different mechanisms of imposex induction, depending on whether the exposure is TBT or testosterone. In TBT-dosed animals, APGWamide levels were elevated. Even in normal TBT-dosed females, levels were elevated, although imposex was not yet induced. In TBT-dosed imposex snails, APGWamide levels were higher than in any other dose group. In testosterone-treated snails, there were no changes in APGWamide levels, but imposex was induced. It is possible that testosterone acts via a different mechanism than TBT. For the transferred animals, both males and females had significantly higher levels of APGWamide than their respective controls. It is clear that APGWamide is upregulated significantly in these transferred animals, and data analysis for imposex animals from this transfer is ongoing.



**Figure 1.** APGWamide levels in snail homogenates as analyzed by Western Blotting. Control = vehicle injected; TBT = 20 ng tributyltin; testosterone = 500 ng; transfers = snails transferred from pristine site to high imposex site. Significance by ANOVA followed by post-hoc Tukey test. Where there are no error bars, n = 1. Otherwise, n = from 5 to 20. Letters above bars indicate no significant differences.

## Endocrinology of Sex Determination in a Crustacean and Its Disruption by Biorational Insecticides

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Terpenoid hormones are one of the two major classes of non-peptide hormones (ecdysteroids being the second) found in insects and crustaceans. Juvenile hormone is the predominant terpenoid hormone of insects, while methyl farnesoate is produced by crustaceans. These hormones are similar to the retinoid hormones found in vertebrates and have major roles in development and reproduction. The objectives of this study were to: (1) evaluate the role of methyl farnesoate in sex determination in the crustacean *Daphnia magna*, and (2) investigate the ability of insecticides that act as juvenile hormone analogs in target species to disrupt methyl farnesoate-regulated developmental processes. Chronic exposure (3 weeks) of small daphnid populations to concentrations of methyl farnesoate dramatically increased the production of male progeny with a lowest observed effect concentration (LOEC) of 80 nM. Acute exposure (24 hours) of individual maternal daphnids to methyl farnesoate during the period of late ovarian oocyte maturation caused these oocytes to develop exclusively into males. These results indicate that methyl farnesoate is responsible for programming oocytes to develop into male offspring.

The insecticidal juvenile hormone analogs methoprene and pyriproxyfen next were evaluated for their ability to mimic the effect of methyl farnesoate on sex determina-

tion of offspring. Both compounds increased male progeny production in small populations after chronic exposure with LOECs of 32 nM and 0.39 nM, respectively. Several non-juvenile xenobiotics were similarly evaluated and had no effect on sex determination. As with methyl farnesoate, the juvenile hormone analogs determined sex of offspring when exposure occurred during the sensitive period of oocyte development. Daphnids were exposed to binary mixtures of methoprene-methyl farnesoate or pyriproxyfen-methyl farnesoate to determine if these chemicals conformed to a model for concentration addition (indicative of same mechanism of action) or independent joint action (indicative of separate mechanisms of action). Although both mixtures conformed more to the concentration additive model, some degree of synergism was detected between the insecticides and the crustacean hormone. In conclusion, this research project found that: (1) the crustacean terpenoid hormone methyl farnesoate determines sex in daphnids, (2) biorational insecticides with juvenile hormone activity can mimic the effects of methyl farnesoate, and (3) the mechanism of action of this effect is similar for both the hormone and the insecticides. Future studies will evaluate the relationship among endogenous methyl farnesoate levels, reproduction, and sex of offspring as well as elucidate the mechanism of synergism between these insecticides and methyl farnesoate.

## Cloning and Characterization of Estrogen Receptor $\alpha$ , $-\beta_a$ , and $-\beta_b$ in Zebrafish: Differential Expression During Development and Effects of Steroids and Environmental Chemicals

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Estrogen actions on growth, differentiation, and proliferation of a range of tissues, cell types, and life stages are mediated chiefly through ligand-dependent transcription factors termed estrogen receptors (ER). Previous studies in this laboratory demonstrated that estrogen and estrogen-like chemicals upregulate expression of the predominant aromatase isoform (P450aromB) in fish brain, and further identified estrogen response elements in the 5'-flanking region of the P450aromB-encoding gene (*cyp19B*). To obtain ER for use as reagents in transcriptional analysis and localization studies, and to determine whether estrogenic effects on expression of P450aromB and other neural genes could be mediated in part by changes in ER expression, a stepwise polymerase chain reaction (PCR) cloning strategy was used to isolate the cDNA(s) encoding ER in zebrafish liver. Three distinct ER isoforms were identified. Based on sequence and domain comparisons and phylogenetic analyses, these were termed ER $\alpha$ ,  $-\beta_a$ , and  $-\beta_b$  to conform to the zebrafish nomenclature guidelines. Northern analysis revealed that transcript numbers and sizes are gene- and tissue-specific. As shown by reverse transcriptase PCR/Southern transfer

analysis, ER $\alpha$ ,  $-\beta_a$ , and  $-\beta_b$  were found to be widely expressed in neural and nonneural tissues, but relative levels and ratios of the different subtypes differed by tissue type.

Although mRNAs encoded by all three ER genes were detectable in unfertilized eggs—signifying maternal transfer—and embryonic transcription had an early onset (12–24 hour postfertilization, hpf), each had a distinct developmental profile in subsequent stages (24–68 hpf) and was differentially affected by exposure of embryos to estrogens, androgens, and known or suspected endocrine disruptors (e.g., bisphenol-A, 2,3,7,8-tetrachlorodibenzo-p-dioxin, polychlorinated biphenyls, atrazine). It was concluded that reported endocrine-disrupting effects of chemicals in laboratory species, and effects of pollutant mixtures on reproduction and development of wildlife, could be due in part to altered patterns of ER expression rather than direct binding to existing ER. Because 0–48 hpf embryos were used as a screening system in these initial studies, further research is required to determine chemical effects on ER at other life stages and in specific tissue types.

## Effects of Perfluorooctane Sulfonate on Thyroid Hormone Status in the Rat and Mouse

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Perfluorooctane sulfonate (PFOS), a compound used in the manufacture of surfactants and insecticides, is a ubiquitous environmental contaminant that recently has been found to be a developmental toxicant in laboratory rodents. The focus of this work is to ascertain if alterations of thyroid status are involved in PFOS toxicity. Timed-pregnant, Sprague-Dawley rats were given 1, 2, 3, or 5 mg/kg/day PFOS/K<sup>+</sup> by oral gavage from gestational day (GD) 2-21; controls received 0.5 percent Tween-20 vehicle (1 mL/kg). Blood samples were collected from the dams on GD 7, 14, and 21 and from the pups throughout postnatal development. Serum thyroid-stimulating hormone (TSH), total and free T4, and T3 levels were determined by radioimmunoassay at all doses in the dams and up to the 3 mg/kg dose in the offspring. Total and free T4 as well as T3 levels in PFOS-treated dams were significantly lower at all time points in comparison to controls, but the TSH levels were not affected. Hypothyroxinemia was observed in the PFOS-exposed pups during postnatal development. Decreased rates of body weight gain in the dams during gestation and the offspring during postnatal development also resulted from this exposure. Because thyroid hormone lev-

els are known to fluctuate with pregnancy, this study was extended to nonpregnant adult rats. Ninety day-old male and female rats received either vehicle control or 5 mg/kg PFOS daily, and were sacrificed after 3 or 20 days of chemical exposures. Trunk blood was collected, and serum thyroid hormone levels were determined.

As seen in the pregnant cohort, the results indicated a reduction of T4 in the PFOS-treated rats (45% or 15% of controls after 3 or 20 days of exposure, respectively) with an absence of feedback elevation of TSH. In fact, the TSH levels of the nonpregnant rats appeared to be lower in the 5 mg/kg group than in controls, at both time points. Additionally, under similar exposure conditions, thyrotoxic effects of PFOS also were seen in the pregnant mouse, although the extent of this hormonal imbalance is less than the rat. Thus, these results suggest that PFOS can be regarded as an environmental endocrine disruptor. However, the underlying mechanisms for its particular profile of hormonal disruption and the potential alterations of physiological functions related to thyroid imbalance warrant further investigation.



## Anti-Androgenic Effects of Vinclozolin on Male Rats Are Partially Attenuated by Testosterone Propionate

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Sexual differentiation to the male phenotype is dependent on activation of the androgen receptor (AR) by androgens during fetal development. The AR inhibitor vinclozolin (V) administered to the rat dam on gestational days (GD) 14-19 compromises masculine development of the male offspring, resulting in nipple formation, cleft phallus, ectopic testes, and reduced prostate, seminal vesicle, and levator ani/bulbocavernosus (LA/BC) weights. Testosterone propionate (TP) administered to the dam during the same gestational period does not affect the male offspring, but masculinizes female offspring. Because both androgenic and anti-androgenic chemicals exist in the environment and the effects of combinations of chemicals are unknown, the study of combinational exposure to these chemicals is important for risk assessment.

In the current study, this research project sought to determine whether co-administration of TP with V attenuates the action of V on sexual development of the male. Sprague-Dawley rats were dosed on GD 14-19 with corn oil (vehicle; 2.5 mL/kg; oral gavage), TP (1 mg/0.1 mL/rat; subcutaneous), V (200 mg/kg; oral gavage), or V+TP. Male offspring were monitored through-

out life and necropsied on postnatal day (PND) 170-186. Litter size on PND 2 was reduced significantly only by V+TP (5.6;  $p < 0.001$ ), although sex ratio was not affected in any treatment group. Consistent with previous results, V reduced anogenital distance (AGD) on PND 2 ( $p < 0.0001$ ); induced nipples (mean  $n = 11.68$ ); induced malformations such as cleft phallus (95%), vaginal pouch (84%), and ectopic testes (59%); and reduced prostate, seminal vesicle, and LA/BC weights in male offspring. TP alone had no effect on any of these endpoints. Co-administration of TP with V significantly reduced the number of nipples induced by V ( $n = 9.50$ ;  $p < 0.005$  compared to V) and reduced the incidence of malformations induced by V (cleft phallus = 75%,  $p < 0.01$ ; vaginal pouch = 48%,  $p < 0.05$ ; ectopic testes = 12.5%,  $p < 0.01$  compared to V). Conversely, V+TP failed to reverse the reduction in AGD or restore weights of ventral prostate, seminal vesicle, and LA/BC reduced by V. It was concluded that co-administration of TP with V at the doses used attenuates some of the anti-androgenic effects induced by V during fetal development in male rat offspring. Future work may investigate the differences in protein expression in these tissues that may be responsible for the difference in response.

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