Metabolic Androgenization of Invertebrates by Endocrine-Disrupting Chemicals

Project Scope

Significant evidence has accumulated over the past decade that indicates some environmental chemicals can interfere with animal endocrine systems. These endocrine disrupting chemicals (EDCs) can affect various endocrine-regulated physiological processes. Of particular concern is the potential exposure of the embryo/fetus to EDCs that can alter development and cause subsequent deficits in growth, maturation, or reproduction. The ecological significance of this mode of toxicity has undergone significant investigation with various vertebrate species; however, little research has focused upon chemical-induced endocrine disruption in invertebrates.

The masculinization of female gastropods (e.g., snails) and crustaceans (e.g., crabs, shrimp) has been documented worldwide. Among gastropods, this condition has been causally associated with the environmental contaminant tributyltin, which until recently was used as an antifouling agent in boat hull paint. Causality has not been conclusively established among crustaceans, though correlative evidence suggests that environmental pollution is contributing to masculinization.

There were two primary objectives for this research project:

- Elucidate the mechanism by which tributyltin elevates testosterone levels in the mud snail (*Ilyanassa obsoleta)*. Elevated testosterone levels in female mud snails is considered responsible for the pseudohermaphroditism (i.e., partial masculinization) in these organisms.
- Identify mechanisms by which environmental chemicals (i.e., juvenoids) could masculinize the crustacean *Daphnia magna*. A juvenoid is a substance that disrupts larval development by mimicking a natural hormone.

Grant Title and Principal Investigator

Metabolic Androgenization of Invertebrates by Endocrine-Disrupting Chemicals

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Key Findings

- The biotransformation of testosterone was definitively characterized in both the mud snail and the water flea, and aspects of testosterone metabolism unique to these groups were discovered.
- Tributyltin tin was found to elevate free testosterone levels in the mud snail at environmentally-relevant exposure concentrations through effects on testosterone esterification.
- Juvenoid EDCs were found to alter sex determination in daphnids.
- Ecdysteroids were found to be important in orchestrating discrete processes in early and late embryo development of daphnids.
- EDCs interfered with endogenous juvenoid and ecdysteroid hormone regulatory pathways in *Daphnia magna* in ways that can inhibit embryo development and reduce fecundity of exposed populations.

Project Period: October 1997 to September 2000

Project Results and Implications

Gastropods. To achieve the first objective of this research project, it was first necessary to elucidate the metabolic fate of testosterone in the mud snail. Once the normal processing of testosterone by these organisms was understood, we developed and tested hypotheses regarding the effect of tributyltin on the processes. Unlike all other laboratory species thus far evaluated (e.g., mice), mud snails did not eliminate excess testosterone as polar metabolites. Rather, excess testosterone was stored in the tissues of the snail as fatty acid esters (Figure 1). This esterification was mediated by a microsomal acyl CoA:testosterone acyltransferase (ATAT) enzyme. Experiments were undertaken to evaluate whether this esterification process served to regulate testosterone levels in the snail.

Relevance to ORD's Multi-Year Research Plan

This project contributes directly to two important long-term goals of ORD's MYP: (1) to provide a better understanding of the science underlying the effects, exposure, assessment, and management of endocrine disruptors, and (2) to determine the extent of the impact of endocrine disruptors on humans, wildlife, and the environment.

This study served EPA's long-term goals by first improving our understanding of basic endocrinology in gastropods and crustaceans, specifically: (1) the regulation of testosterone metabolism in mud snails, and (2) juvenoid and ecdysteroid hormone regulatory pathways in daphnids. Once these processes were elucidated, mechanisms by which EDCs may cause masculinization in these organisms (by interfering with those basic endocrine system processes) were identified.

Tributyltin elevated free testosterone levels in mud snails at environmentally-relevant exposure concentrations, and snails impacted by tributyltin exhibited abnormalities in sex differentiation and had reduced fecundity. EDCs (e.g., methoprene, pyriproxyfen, testosterone, fenarimol) interfered with endogenous juvenoid and ecdysteroid hormone regulatory pathways in daphnids in ways that inhibited embryo development and reduced fecundity of exposed populations.

Conversion of testosterone to testosterone-fatty acid esters by the mud snail is illustrated in Figure 1. Biotransformation of testosterone by the mouse is presented for comparison. Lane 1 under 'mud snail' depicts the migration of testosterone in this thin-layer chromatography (TLC) system. Lanes 2 and 3 depict metabolites that were extracted from snail tissues following exposure to [^{14}C]testosterone and resolved by TLC. Presented is an autoradiograph of the TLC plate. While mice, and other species, readily convert testosterone to a variety of polar metabolites that are eliminated through waste products, the mud snail converts testosterone to fatty acid esters that are retained by the organism.

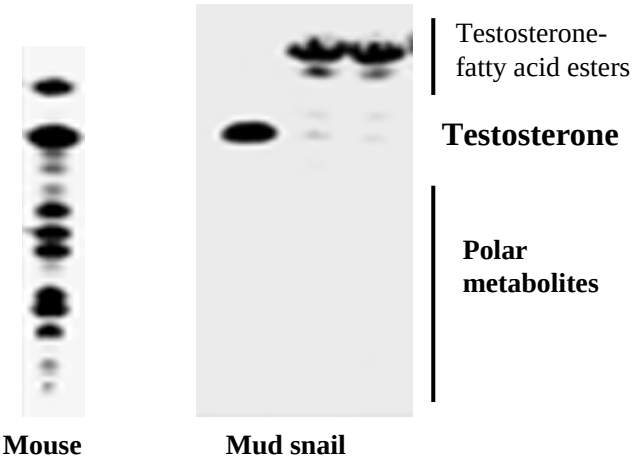

Figure 1. Conversion of testosterone to testosterone-fatty acid esters by the mud snail *I. obsoleta*.

Snails were provided various tributyltin treatments for a range of exposure durations to modulate the level of total testosterone in their tissues. The snails were evaluated for esterified and free (unesterified) testosterone levels. Testosterone ester levels varied in direct proportion to the level of total testosterone in the organisms. In contrast, free testosterone levels remained remarkably constant, despite total testosterone levels in the tissues that ranged several orders of magnitude. These results demonstrated that testosterone esterification stores excess amounts of testosterone, and these stores are drawn upon when testosterone levels are depleted. Thus, snails have developed a remarkably simple, yet effective, means of regulating testosterone levels in their tissues. Results also revealed that free testosterone levels normally increase at the time of sexual differentiation and at the end of reproduction. During these

periods, testosterone levels were higher in males than in females. These surges in free testosterone levels were due largely to decreases in the esterification of testosterone.

These observations led to the hypothesis that tributyltin elevates testosterone levels in snails by interfering with the testosterone esterification process. Concordant with this hypothesis, experimental tributyltin exposure of mud snails in our laboratory decreased testosterone esterification, resulting in an elevation of free testosterone. This effect appears to be due to direct competitive inhibition of the ATAT enzyme by tributyltin.

Snails were sampled from a tributyltin-contaminated site (Bald Head Marina) and a control site (Bald Head Creek) located along the North Carolina coast at various times during their reproductive cycle, and were monitored for sexual development, reproduction, and testosterone homeostasis. Consistent with laboratory studies, snails collected from the tributyltin-contaminated site generally had lower testosterone ester levels and higher free testosterone levels compared with snails from the non-contaminated site. Snails from the contaminated site also exhibited abnormalities in sex differentiation and had reduced fecundity. Among snails sampled from the tin-contaminated site, the surge in free testosterone levels associated with sexual differentiation was attenuated and occurred earlier than in snails from the non-impacted site. These field observations confirmed that tributyltin elevates free testosterone levels by interfering with testosterone esterification. Tributyltin also appears to disrupt seasonal surges in free testosterone that may be instrumental in regulating sexual differentiation and reproduction.

Crustacea. Physiological mechanisms were identified in which environmental chemicals could cause masculinization in crustaceans (i.e., the development of male sex characteristics in females). A conceptual model also was formulated in which the development of male sex characteristics is dependent upon both organizational events during embryo development when the sex of the embryo is determined and activational events during maturation in which male sex differentiation occurs.

The activational effects of endocrine-active compounds on crustacean sex differentiation were evaluated by exposing immature male and female daphnids (*Daphnia magna*) to endocrine-active compounds (i.e., methoprene, androstenedione, diethylstilbestrol) and by evaluating the rate of development of sex-specific characteristics. These endocrine-active compounds represent good examples of hormone and hormone-like compounds for evaluating sex differentiation. The compounds evaluated did not cause the development of abnormal sex characteristics in either sex. However, several compounds altered the rate of development of sex characteristics in the appropriate sex. For example, the juvenoid methoprene (used as an insecticide) stimulated the development of the female-specific abdominal differentiation, and the androgen androstenedione stimulated elongation of the first antennae, a male-specific characteristic.

Continued investigation into activational effects of chemicals on male sex determination revealed that the juvenoid methoprene significantly altered sex ratios of offspring in favor of males. This effect was confirmed with two additional juvenoids (methyl farnesoate and pyriproxyfen). Methyl farnesoate was determined to be the endogenous male-sex determining hormone in daphnids, and environmental juvenoids (i.e., methoprene, pyriproxyfen) have the ability to disrupt sex ratios in daphnid populations by acting as methyl farnesoate mimics. Maternal daphnids exposed to the juvenoids sometimes produced sexually ambiguous offspring (i.e., pseudohermaphrodites), although the frequency was too low to ascribe any significance to the occurrences. Concentrations of pyriproxyfen were found that elicited no adverse effect on maternal organisms, but caused them to produce only male offspring. This effect of pyriproxyfen occurred at many orders of magnitude below the exposure levels known to be acutely toxic to daphnids or to elicit effects in other species.

Several endocrine-active compounds also were evaluated for organizational effects during embryo development. These experiments initially focused on potential environmental contaminants that were postulated to be androgenic. Steroidal androgens (i.e., testosterone and androstenedione) and chemicals that inhibited the elimination of testosterone (i.e., 4-nonylphenol, propiconazole, and fenarimol) elicited developmental abnormalities in embryos (e.g., early partial developmental arrest, incomplete development of antennae and shell spines). However, these compounds did not alter sex ratios of offspring in favor of males, nor did they masculinize female offspring. Further analyses confirmed that

some of these compounds (i.e., testosterone and fenarimol) elicited developmental abnormalities by functioning as antiecdysteroids. Ecdysteroids serve to direct major embryonic developmental transitions. Two mechanisms of antiecdysteroidal activity were discerned: lowering of ecdysteroid levels and ecdysteroid receptor antagonism. Mechanistic studies revealed that mixtures of chemicals that elicit antiecdysteroidal effects on developing embryos by these two differing mechanisms can result in synergistic toxicity, with greater than additive decreases in population fecundity.

Implications of results and future research. These experiments have provided major insights into the regulation of testosterone in the mud snail. The elucidation of important processes that contribute to the maintenance of testosterone homeostasis in this species represents a significant advancement in understanding the basic endocrinology of gastropods. This project demonstrated tributyltin-induced modulation on testosterone biotransformation in the mud snail. Elucidation of the effect of tributyltin on normal testosterone homeostasis in snails has led to an increased mechanistic understanding of tributyltin as an EDC in gastropods. Demonstration in laboratories of the adverse effects of tributyltin on gastropods and other species contributed to a ban on tributyltin-based paints that was ratified by the International Maritime Organization, effective January 1, 2003. Observations in our studies identified a target of tributyltin on testosterone regulation in the mud snail. More information on the precise mechanism of masculinization of females exposed to tributyltin remains to be elucidated, and this understanding is necessary to ensure that antifoulants developed in the future do not have adverse impacts on non-target organisms.

EDCs interfered with two hormonal regulatory pathways in the crustacean *Daphnia magna* that could reduce fecundity of exposed populations. Ecdysteroid hormones are critical to normal crustacean embryo development, and several EDCs had antiecdysteroidal activity. Juvenoid hormones (e.g. methyl farnesoate) are sex determinants in daphnids. Environmental juvenoids, some of which have widespread application as insecticides (e.g., methoprene and pyriproxifen) can mimic the action of methyl farnesoate and alter sex ratios among daphnid offspring. Clearly, such effects would have serious consequences in similarly exposed field populations. Other environmental juvenoids need to be evaluated for organizational toxicity such as effects on sex determination.

In addition to the individual effects of the antiecdysteroids and juvenoids on crustacean fecundity, several instances of synergistic interactions among the chemicals that we evaluated were observed. Future studies will further elucidate such synergism, particularly between antiecdysteroidal chemicals and juvenoid chemicals. Research suggests that interactions occur between chemicals of these two mechanistic classes that would result in significantly impaired reproductive capacity of exposed crustacean populations.

Investigators

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For More Information

http://www.tox.ncsu.edu/

NCER Project Abstract and Reports:

http://cfpub2.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/180/report/F