Endocrine Disruption in Marine Gastropods by Environmental Chemical Mixtures

Project Scope

The occurrence of imposex, imposition of male sexual characteristics on female snails, has been extensively documented throughout the world. Tributyltin (TBT) and other organotins have been causally linked to imposex induction at levels as low as 2 ng/L. Females exposed to TBT grow accessory sex organs (ASO), including sperm ducts, seminal vesicles, external sperm grooves, and 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 mollusks; the other is based on the vertebrate model of steroid hormone regulation.

TBT, used for years as an antifouling agent in boat hull paints (until banned in 2003 by the International Maritime Organization), is rarely the sole contaminant in harbors. Other contaminants such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs), which are known to interact with the steroid-hormone system in vertebrates, also are found in harbors. The goal of this study was to determine the biochemical mechanisms responsible for TBT-induced imposex in mud snails (*Ilyanassa obsoleta*) and examine the interrelationship between TBT and other environmental xenobiotics in the development of these sexual abnormalities.

The main objectives of this research were to:

- Investigate the biochemical and/or molecular mechanisms by which TBT alters the regulation of steroid hormones and neurohormones to induce imposex in *Ilyanassa obsoleta*;
- Determine the effects of other environmental contaminants (i.e., 3-methylcholanthrene [3-MC]) on TBT's ability to induce imposex; and
- Determine whether the mechanisms identified in laboratory studies on imposex are active in field-induced organisms.

Grant Title and Principal Investigator

Endocrine Disruption in Marine Gastropods by Environmental Chemical Mixtures

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Key Findings

- Tributyltin (TBT) induces significant levels of imposex (imposition of male sexual characteristics) in female mud snails, which is lessened if females are simultaneously exposed to 3-methyl-cholanthrene (3-MC).
- Injection of the neuropeptide hormone APGWamide into female snails resulted in the initiation of penile bud formation indicating that this peptide is a strong candidate for the penile morphogenic factor (PMF) in mud snails.
- Exposure to TBT causes an increase in the level of APGWamide expression in the tissues of snails. This increase was evident in female (least), imposex female (intermediate), and male (highest) snails exposed to TBT in the laboratory or through environmental exposure.
- Inhibition of cytochrome P450 aromatase activity is not responsible for the induction of imposex but may play a role in the maintenance or severity of penile formation in female snails.
- Inhibition of cytochrome P450-mediated steroid biosynthesis is not responsible for the induction of imposex, but steroids may play a role in the maintenance or severity of penile formation in female snails.
- In summary, TBT likely stimulates the release of PMF (identified as APGWamide) to induce imposex in females (resulting in the growth of the accessory sex organs [ASO] and the production of steroids). Steroids then act as part of a hormonal feedback loop to maintain the ASO.

Project Period: September 1999 to August 2002

Relevance to ORD's Multi-Year Research Plan

This project contributes to the first long-term goal of the ORD's MYP (LTG-1): to provide a better understanding of the science underlying the effects, exposure, assessment, and management of endocrine disruptors.

Results of laboratory and field studies elucidated the biochemical mechanisms responsible for TBT-induced imposex in mud snails (*Ilyanassa obsoleta*). This research indicated that neither the inhibition of cytochrome P450 aromatase nor the inhibition of the P450-mediated steroid biosynthesis is responsible for imposex induction in mud snails. Instead, it was proposed that TBT stimulated the release of the penile morphogenic factor (PMF) in mud snails (identified as APGWamide) to induce imposex in females. Release of the PMF leads to the growth of accessory sex organs (ASO) and the production of steroids. These steroids function in a positive feedback loop that helps to maintain ASO. These studies also demonstrated that co-exposure of TBT- dosed snails to certain PAHs (e.g., 3-MC) decreases the level of imposex induction. Overall, this research provides valuable data linking the exposure of gastropods to TBT and certain PAHs to sexual development and possible effects at the population level.

Project Results and Implications

Imposex Induction

Laboratory. Thirty female mud snails were exposed in aqueous solution for 45 days to 20 ng/L TBT, 10 nM 3-MC, or a mixture of 20 ng/L TBT + 10 nM 3-MC. The control group was exposed to the same volume of the dissolving vehicle (ethanol) as the test groups (20 µL[ethanol solution]/L[sea water]). At the end of the exposure period, the snails were removed from their shells and the incidence of imposex was determined. Results indicated that exposure of snails to TBT at concentrations of 20 ng/L water induced imposex (determined by the percentage of females bearing a penis or penal bud) in approximately 40 percent of female mud snails compared to approximately 6 percent in controls, and that co-exposure to 10 nM 3-MC reduced imposex incidence to approximately 22 percent. These results confirmed the following: (1) TBT does induce imposex in female snails; (2) 3-MC does not induce imposex; and (3) co-exposure of 3-MC with TBT decreases the ability of TBT to elicit imposex induction (see Figure 1). The mechanism by which 3-MC co-exposure reduces imposex formation currently is being investigated.

As shown in Figure 1, seasonal variability was observed in the induction of imposex following exposure to these contaminants. Imposex was induced in a higher proportion of female snails exposed to TBT during the non-breeding season (late summer/fall) than in females exposed during the breeding season (winter/spring). This latter result indicates that preceding hormonal status and/or environmental factors, such as temperature, photoperiod, tidal cycle, nutritional status, etc., may play a role in the manifestation of endocrine effects or susceptibility to endocrine disruption in marine gastropods.

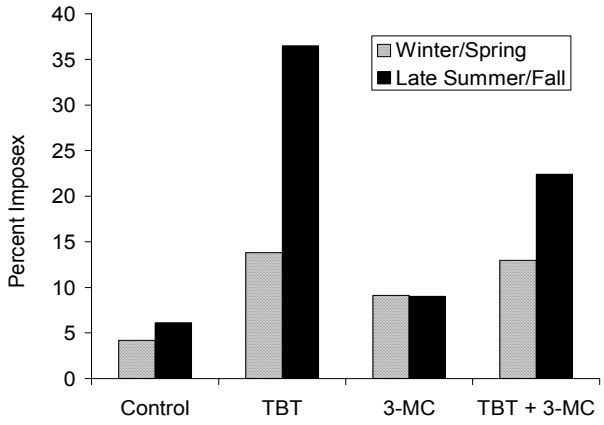

Figure 1. Induction of imposex in female mud snails following exposure to TBT and a PAH. Thirty female mud snails were exposed for 45 days to the control solvent (ethanol), 20 ng/L TBT dissolved in ethanol, 10 nM 3-methylcholanthrene (3-MC), or a mixture of 20 ng/L TBT and 10 nM 3-MC. (Results given are an average of 4 experiments from each season.)

This study also showed that exposure of mud snails to TBT at concentrations of 20 ng/L decreased the number of egg capsules laid by females to approximately 50 percent of the controls. In contrast, exposure to 3-MC or to the TBT/3-MC mixture increased the number of egg capsules laid relative to controls by 50 to 60 percent. This indicates that steroid-like PAHs, which can function as environmental estrogens, can enhance reproductive output of the snails. In a separate study, the male mud snails exposed to TBT at 20 ng/L for 45-days exhibited an increased penal length that was 2.1-times that of the control males.

***Field*.** Four thousand snails, presumably including an equal number of males and females, were collected from a reference (control) site at Bird Shoals in the Rachael Carson Estuarine Research Reserve, NC. Snails were placed in one of two Mylar mesh cages. The first cage was anchored at the control site; the second cage was placed at a site identified as having a high imposex rate (~87 percent; Morehead City, NC). The sediments were not analyzed for TBT at this site, but it does contain a boat re-fitting shop that had used TBT-based paints for many years. Cages remained in place for three months (September – November). Following the three-month exposure, induction of imposex at the Morehead City site (~25 percent) was significantly higher than the rate of imposex at the control site (~8 percent). In addition, male snails transferred to the test site exhibited an 8-fold increase in penis length compared to males caged at the control site.

TBT Effects on Neuropeptide Hormone Expression

The first model of imposex induction centers on the concept that TBT acts on the neural ganglia of snails to alter the expression of neuropeptide hormones. Through this mechanism, TBT is hypothesized to control the expression/activity of the putative penile morphogenic factor (PMF), a neuropeptide that controls the production of male accessory sex organs in gastropods.

***Laboratory*.** Four different molluskan neuropeptides – APGWamide, FMRFamide, LSSFVRIamide, and conopressin – were investigated for their ability to induce penile development during a two-week exposure period. Snails were injected every other day for 14 days with one of the four peptides, or 0.2 or 20 ng TBT/animal (dissolved in 5 µL ethanol) as a positive control, or 5 µL ethanol as the negative control. Results of these studies demonstrated high levels of imposex (approximately 80 percent) in response to injections with APGWamide. Percent imposex induction for the other neuropeptides ranged from approximately 7 to 20 percent; the high dose of TBT resulted in approximately 55 percent imposex. Thus, APGWamide was tentatively identified as the PMF in mud snails. Follow-up studies indicated that exposure to TBT likely causes an increase in the expression of this peptide in the tissues of male, female, and imposex snails.

Field. The level of expression of APGWamide in the tissues of the cage-control and cage-transferred snails (see Imposex Induction study above) were examined in the field. Whole snail tissues from control female, imposex female, and control males were sectioned and mounted onto slides and were visually examined by microscope. The peptide was identified using a 1:25 dilution of the polyclonal anti-APGWamide antibody. APGWamide was visible in the ganglia of the imposex females (cage transferred to the contaminated site) and the males from the control site. The peptide was not visible in any of the tissues from control site females. This indicates that the peptide APGWamide is a strong candidate for the PMF in the mud snail and that this peptide is up-regulated in response to TBT exposure. The level of peptide expression was significantly higher in the cage-transferred snails compared to the cage-control or the laboratory-exposed animals. The increased expression may be related to the higher TBT exposures in the environmental samples.

TBT Effects on P450 Aromatase Activity

In the second model of imposex induction, TBT is proposed to function as a noncompetitive inhibitor of cytochrome P450 metabolism. Cytochrome P450 aromatase is the key enzyme responsible for the conversion of testosterone to estradiol in snails. Inhibition of this enzyme would theoretically cause an increase in testosterone levels that could lead to masculinization of the female mud snails.

Laboratory. To test the above hypothesis, 360 female snails (90 snails per treatment) collected from the Rachael Carson Estuarine Research Reserve were exposed for 45 days in the laboratory during the

breeding and non-breeding seasons as follows: (1) controls, no exposure; (2) 20 ng/L of TBT; (3) 10 nM 3-MC, or (4) both compounds (20 ng/L TBT + 10 nM 3-MC). A summary of aromatase activity in the control and treated groups is provided in Table 1. Exposure to both TBT and/or 3-MC inhibited the level of cytochrome P450 aromatase activity in snails. As shown in Table 1, aromatase activity was higher in non-breeding than in breeding female snails. However, TBT inhibited P450 aromatase activity in both the breeding and non-breeding mud snails, indicating no specific difference associated with reproductive status.

Table 1. Summary of P450 Aromatase Activity (pmol/mg-hr) in Female Mud Snails

Laboratory Samples	Breeding	Control	14.767 (+ 2.81)
		TBT only	9.543 (+ 1.13)
		3-MC only	6.049 (+ 0.916)
		TBT + 3-MC	9.967 (+ 6.456)
	Non-breeding	Control	30.18 $\pm$ 12.3
		TBT only	14.6 $\pm$ 7
		3-MC only	14.3 $\pm$ 11.1
		TBT + 3-MC	14.2 $\pm$ 7.8
Field Samples	Low Imposex Area (Bird Shoals)	Normal Female	0.224 (+ 0.137)
		Male	0.454 (+ 0.263)
	High Imposex Area (Morehead City)	Normal Female	0.0828 (+ 0.034)
		Imposex Female	0.0752 (+ 0.047)

Field. Snails were also examined for their P450 aromatase activity in the field. Whole-cell homogenates were prepared from the abdominal tissues, and the levels of P450 aromatase activity were evaluated. As shown in Table 1, female snails from the low-imposex (reference) site (Bird Shoals) exhibited a higher level of cytochrome P450 aromatase activity than females from the high-imposex area (Morehead City). Also as shown in Table 1, aromatase activity was lower in both morphologically "normal" and "imposex" females from the Morehead City site compared with the controls. This indicates that inhibition of aromatase activity, presumably due to exposure to contaminated sediments, is not sufficient by itself to induce imposex. P450 aromatase activity was notably lower in field versus laboratory samples. This is most likely because TBT levels are probably higher in the field, and there are other compounds (including PAHs and pesticides) present in the field that can also depress aromatase activity.

TBT Effects on Steroid Biosynthesis

A theory favored by many researchers was that TBT blocks aromatase activity (as observed above) and thereby inhibits conversion of testosterone to estradiol. This results in a buildup of testosterone in the tissues and masculinization of the female snails. An alternative theory, tested in the following experiments, is that if TBT is really a non-competitive inhibitor of P450 aromatase, then it should also inhibit the production of other steroids whose synthesis depends on P450.

Laboratory. The role of TBT on P450-mediated steroid metabolism was evaluated by examining testosterone and androstenedione metabolism, as well as measuring the levels of testosterone, androstenedione, and progesterone in 288 female adult snails following a 45-day exposure to 20 ng/L TBT, 10 nM 3-MC, or the combination 20 ng/L TBT+ 10 nM 3-MC. As shown in Table 2, TBT exposure for 45 days decreased the levels of free steroids produced by breeding snails. The steroid levels of non-breeding snails were assayed, but levels were at or below the limit of detection.

These findings are consistent with reduced steroid conversion due to TBT exposure and thus less testosterone to be converted. A build-up of testosterone (and masculinization of the female snails) would not readily occur because there is less testosterone being produced. Therefore, the inhibition of P450-mediated steroid biosynthesis is *not* responsible for imposex induction.

Table 2. Effect of Chemicals on Steroid Levels in Female Mud Snails (*I. obsolete*) following a 45-day Exposure

Free Steroid Levels (Breeding Snails)			
	Progesterone(ng/mL)	**Androstenedione(nmol/L)**	**Testosterone(pmol/L)**
Control	0.593 (+ 0.21)	2.560 (+ 2.32)	0.440 (+ 0.88)
TBT only	0.283 (+ 0.20)	0.530 (+ 1.10)	ND
3-MC only	Not detected	0.044 (+ 0.06)	3.610 (+ 7.23)
TBT + 3-MC	0.087 (+ 0.001)	Not detected	Not detected

Field. *In vivo* metabolism of pregnenolone, progesterone, and androstenedione in female, imposex female, and male cage-transferred snails was examined. Female snails were incubated overnight (16 hours) with 300 pmol pregnenolone, 4 nmol progesterone, or 200 pmol androstenedione in 35 ppt sterile filtered sea water. The snails were removed from their shells and the whole tissue homogenates were extracted and examined by thin-layer chromatography (TLC). Very few metabolites were produced that could be identified using known standards (androstenedione, 3α or 3β androstanediol, 5α dihydrotestosterone, androsterone, testosterone, 2α or 2β, 6α or 6β, 7α, 16α or 16β hydroxytestosterone). It was readily apparent during examination of the TLC traces that the imposex female snails metabolized each of these steroids in a manner that more closely resembled male snails than female snails.

The aromatase results, along with the steroid biosynthesis results, indicate that P450-mediated steroid conversion is inhibited by TBT and other environmental chemicals such as steroid-like PAHs (e.g., 3-MC). TBT *does* inhibit P450 aromatase activity (testosterone conversion to estradiol) and steroid biosynthesis and *does* cause induction of imposex. 3-MC also inhibits P450 aromatase activity and inhibits steroid biosynthesis, but *does not* cause imposex induction. Thus, inhibition of P450 aromatase activity and steroid biosynthesis, by itself, is not responsible for imposex induction.

Summary

Our research indicates that neither the inhibition of cytochrome P450 aromatase nor the inhibition of the P450-mediated steroid biosynthesis is responsible for imposex induction in mud snails. Co-exposure of female snails to TBT and the environmental contaminant 3-MC reduces the level of imposex induction, but this reduction is not mediated through the steroid metabolic pathway or through an increase in the breakdown of TBT. We propose a mechanism of imposex induction based on the neuropeptide hormone model (Figure 2). PMF, the penis morphogenic factor, tentatively identified by this study as APGWamide, causes the development of male secondary sex characteristics following an external stimulus. This leads to the growth of the accessory sex organs (ASO) and the production of steroids. These steroids then function in a positive feedback loop that helps to maintain these structures. TBT would stimulate the release of PMF to induce imposex in females while testosterone (T) and estrogen (E2) would act on the feedback loop.

Project Implications

Exposure of marine gastropods to environmental mixtures of EDCs has the potential to substantially interfere with normal steroid hormone biochemistry. In addition, TBT acts on the neural ganglia of snails to alter the expression of neuropeptide hormones that control sexual maturation and reproduction. Resulting outcomes include altered sexual characteristics (imposex) and altered steroid metabolism. 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 ASO. TBT appears to stimulate the release of PMF to induce imposex in females, while steroids act on the feedback loop to maintain these structures.

By determining the interrelationship between environmental androgens and anti-androgen/estrogen-like compounds, these studies provide valuable data linking the exposure of organisms to TBT and certain PAHs to disturbances in sexual development and possible effects at the population level.

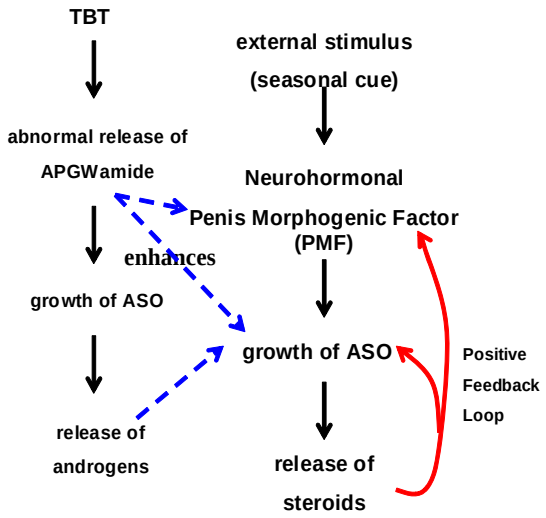

Figure 2. The hypothesized mechanism for imposex induction by TBT. TBT acts on the nervous system, while steroids act on the positive feedback loop which maintains the accessory sex organs

Investigators

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For More Information:

Principle Investigator's Web Page:

http://www.nicholas.duke.edu/people/faculty/mcclellan-green.html

NCER Project Abstract and Reports:

http://cfpub2.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/445/report/0