# The Biocide Tributyltin Reduces the Accumulation of Testosterone as Fatty Acid Esters in the Mud Snail (*Ilyanassa obsoleta*)

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**Imposex, the development of male sex characteristics by female gonochoristic snails, has been documented globally and is causally associated with exposure to the ubiquitous environmental contaminant tributyltin (TBT). Elevated testosterone levels in snails also are associated with TBT, and direct exposure to testosterone has been shown to cause imposex. We discovered previously that the mud snail (*Ilyanassa obsoleta*) biotransforms and retains excess testosterone primarily as fatty acid esters. The purpose of this study was to determine whether TBT interferes with the esterification of testosterone, resulting in the elevated free (unesterified) testosterone levels associated with imposex. Exposure of snails to environmentally relevant concentrations of TBT (≥ 1.0 ng/L as tin) significantly increased the incidence of imposex. Total (free + esterified) testosterone levels in snails were not altered by TBT; however, free testosterone levels increased with increasing exposure concentration of TBT. TBT-exposed snails were given [$^{14}$C]testosterone to measure the production of [$^{14}$C]testosterone–fatty acid esters. The production of testosterone–fatty acid esters decreased with increasing exposure concentration of TBT. These results indicate that TBT elevates free testosterone levels in snails by decreasing the production or retention of testosterone–fatty acid esters. These findings were confirmed among field-sampled snails where individuals collected from a high-tin–affected site exhibited a greater incidence of imposex, higher free testosterone levels, and lower testosterone–fatty acid ester levels when compared with individuals sampled from a low-tin–affected site. Decreased testosterone–fatty acid esterification among TBT-treated snails was not caused by direct inhibition of the acyl coenzyme A:testosterone acyltransferase (ATAT) enzyme responsible for testosterone esterification, nor by suppressed ATAT protein expression. The target of TBT may be a co-contributor to the testosterone fatty esterification process or a factor in the enhanced hydrolysis of the testosterone–fatty acid pool. *Key words:* acyltransferase, fatty acid esters, *Ilyanassa obsoleta*, imposex, invertebrate endocrinology, mud snail, testosterone, tributyltin. *Environ Health Perspect* 111:426–430 (2003). doi:10.1289/ehp.5779 available via *http://dx.doi.org/* [Online 30 October 2002]**

Tributyltin (TBT) is a ubiquitous contaminant in the marine environment that causes a pseudohermaphroditic condition known as imposex in female prosobranch gastropods. Imposex is characterized by the presence of a penis and/or vas deferens in females and has been identified in over 140 species of snails worldwide (Matthiessen et al. 1999). Testosterone is reportedly elevated in imposex individuals and has been causally implicated in the occurrence of this disorder (Bettin et al. 1996; Spooner et al. 1991). Marine prosobranch gastropods are extremely sensitive to TBT contamination, and imposex can be elicited in some species at concentrations of < 1 ng tin/L (Bryan et al. 1987; Gibbs et al. 1988). The increase in testosterone titers with TBT exposure has been attributed both to the inhibition of the aromatase enzyme (CYP19A) (Oehlmann and Bettin 1996; Spooner et al. 1991) and a decrease in the metabolic elimination of testosterone as sulfate conjugates (Ronis and Mason 1996).

The aromatase inhibition hypothesis was developed in response to the observation that testosterone levels were significantly elevated in TBT-exposed dog whelks (*Nucella lapillus*) when compared with controls (Spooner et al. 1991). Increases in testosterone levels following TBT exposure were proposed to be caused by a change in the estradiol:testosterone ratio resulting from the direct inhibition of aromatase by TBT. However, TBT exposure had no effect on estradiol levels in these experiments (Spooner et al. 1991). Although the inhibition of aromatase by TBT remains a possible mechanism of imposex, we are not aware of any direct evidence that TBT inhibits aromatase at environmentally relevant concentrations.

In a study conducted with the periwinkle (*Littorina littorea*), TBT reportedly reduced the metabolic clearance of exogenously administered [$^{14}$C]testosterone as sulfate conjugates (Ronis and Mason 1996). These investigators proposed that reduced clearance of testosterone by TBT-exposed organisms increased accumulation of the hormone. The involvement of this metabolic effect of TBT in imposex was questioned by Oberdörster et al. (1998), who were unable to detect significant sulfate conjugation of testosterone in the mud snail (*Ilyanassa obsoleta*).

Because of the uncertainties regarding the biotransformation and disposition of testosterone by gastropods, we characterized the biotransformation of testosterone in the mud snail (*I. obsoleta*) (Gooding and LeBlanc 2001). Testosterone was not readily eliminated by this species. No production of polar testosterone conjugates was detected, and comparably little hydroxy metabolites or oxido-reduced derivatives were generated. Rather, testosterone was retained largely as nonpolar fatty acid conjugates. Thus, the mechanism by which TBT causes imposex in this species does not involve decreased metabolic elimination of testosterone. The esterification of testosterone to a fatty acid ester as a strategy for steroid regulation reveals novel potential targets of TBT in the development of imposex. TBT could inhibit or suppress the acyl coenzyme A (CoA):testosterone acyltransferase (ATAT) enzyme that converts testosterone to the fatty acid ester, resulting in an increase in free testosterone. In this study we investigated the effect of environmentally relevant concentrations of TBT on the fatty acid esterification of testosterone. We hypothesized that TBT elevates free testosterone levels in the snail by inhibiting the accumulation of testosterone–fatty acid esters.

## Materials and Methods

***Mud snail.*** Snails used in all laboratory experiments were collected at low tide from Bald Head Creek, Bald Head Island, North Carolina, and Bird Shoals in the Rachel Carson Reserve, Morehead City, North

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Carolina. Incidence of imposex from both sites was < 7%. In the laboratory, snails were maintained in 8-L glass aquaria with each aquarium containing 1–2 L reconstituted seawater (35 ± 1 ppt) (Instant Ocean; Aquarium Systems, Mentor, OH) under constant aeration. Thirty-two to 40 snails were housed per tank. Aquaria were maintained at 20°C in a constant temperature incubator (Precision Scientific, Chicago, IL) with a 16-hr light/8-hr dark cycle. Snails were fed Tetramin (Tetra, Werke, Germany) fish flakes *ad libitum*. Water was changed daily, and snails were acclimated to laboratory conditions at least 2 weeks before experiments. A comparison of snails collected from a high- and low-tin–affected site was performed with snails collected from Bald Head Creek (low-tin–affected) and Bald Head Island Marina (high-tin–affected), Bald Head Island, North Carolina.

***Tributyltin exposures.*** Laboratory exposures to tributyltin (chloride) (Aldrich, St. Louis, MO) were maintained in the same manner as the holding conditions described above. Female mud snails are typically larger than males, so larger individuals were selected for exposures to skew the sex ratio toward females. Snails were exposed for 3 or 6 months to environmentally relevant levels of TBT (0.10, 1.0, and 10 ng/L, nominal concentrations, as tin). Individuals were randomly selected for each treatment group. TBT exposure solutions were replaced daily. Stock solutions of TBT chloride were prepared in dimethylsulfoxide (DMSO) (Fisher, Pittsburgh, PA). The concentration of DMSO in all treatments, including controls, was 0.013%.

***Imposex assessments.*** After 3- or 6-month exposures to TBT, sex of the snails was determined, and females were evaluated for imposex. Snails were removed from the shell to reveal the reproductive tract. Males were distinguished by the presence of seminal vesicles and red-orange gonad (Smith 1980). Females were identified by the presence of a white egg capsule gland, albumen gland, and cream-colored gonad (Smith 1980). Imposex was established by the presence of a penis on females located dorsal to the right tentacle. The incidence of imposex in field populations was evaluated using the same criteria.

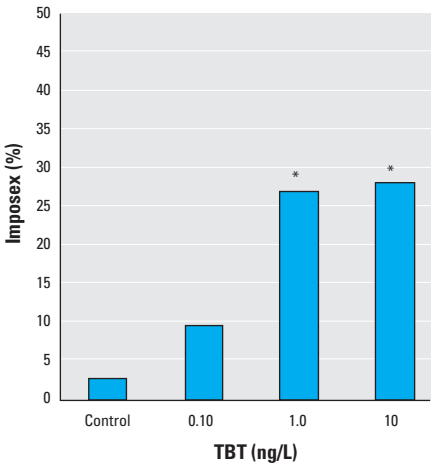


**Figure 1.** Concentration–response relationship for the induction of imposex after TBT exposure. Data are presented as the percentage of imposex females following a 6-month laboratory exposure to TBT ($n$ = 31–41 snails per treatment).
*Significant difference from controls, $p \leq 0.05$.

***Testosterone quantification.*** Tissue from each snail was homogenized for 30 sec in 1.0 mL ethanol using a Tissue Tearor homogenizer (Biospec Products, Bartlesville, OK). Homogenate was stored at –20°C for at least 24 hr, then testosterone and its metabolites were extracted from the tissues with 3.0 mL ethyl acetate. Sample extract was divided into two equal volumes. Aliquots were evaporated under nitrogen, and one aliquot was resuspended in 250 µL 0.05 M phosphate buffer (pH 7.6) for free (unesterified) testosterone quantification. Total (unesterified + esterified) testosterone was determined by saponification of the second aliquot in 1.0 mL methanol containing 1.0% potassium hydroxide (modified from Addo et al. 1989). Samples were incubated for 3 hr at 45°C in an atmosphere of nitrogen. Following saponification, 4.0 mL deionized water was added to each tube and the sample was extracted with 3.0 mL methylene chloride. Extracts were evaporated under nitrogen, resuspended in 1.0 mL 0.05 M phosphate buffer (pH 7.6), and measured for testosterone by radioimmunoassay (RIA) (Diagnostic Products Corp., Los Angeles, CA). No corrections were made for extraction and saponification efficiencies that were ≥ 93%. Testosterone was measured in the samples according to the protocol provided with the RIA kit. The RIA was validated for use in snail extract using criteria of parallelism as we have described previously (Parks et al. 1999). Intra- and interassay coefficients of variation were < 10%. Standard curves were routinely generated using both the standards provided in the RIA kit and standards prepared in the phosphate buffer. Both curves yielded comparable results. Testosterone–fatty acid ester in tissue samples was calculated as total testosterone minus free testosterone. Most (> 70%) and perhaps all of the conjugates of testosterone generated by the mud snail are fatty acid esters (Gooding and LeBlanc 2001). Testosterone levels were normalized to the wet weight of the tissues extracted.

***[$^{14}$C]testosterone–fatty acid esterification.*** After 3-month exposures to TBT, the ability of snails to convert [$^{14}$C]testosterone to [$^{14}$C]testosterone–fatty acid ester was evaluated. Snails (eight per treatment) were individually exposed to media containing [$^{14}$C]testosterone (150,000 dpm, 53.6 mCi/mmol; NEN Products, Dupont, Boston, MA) for 8 hr. Each snail was exposed in a 13 × 100 mm borosilicate glass tube containing 3.0 mL media. An inverted Pasteur pipette was placed in each tube to keep snails from climbing out of the media. At the conclusion of the exposure, snails were sexed, imposex status was noted, and wet tissue weight was recorded to the nearest 0.01 g. Tissues were stored in individual microcentrifuge tubes at –80°C until analysis. Tissue extracts were prepared as described for testosterone quantification. The extract was dried under nitrogen and then spotted onto 20 × 20 cm aluminum-backed silica thin layer chromatography (TLC) plates (Whatman Ltd., Maidstone, Kent, England). [$^{14}$C]testosterone:fatty acid ester was resolved from free testosterone using a mobile phase of hexane:methyl-*tert*-butyl ether:formic acid (80:20:2 v/v/v). The relative amounts of ester and free testosterone in tissue were quantified by electronic autoradiography. (Instant Imager; Packard, Downers Grove, IL). Snail wet weights among treatment groups were statistically indistinguishable [analysis of variance (ANOVA), $p$ = 0.63], and results were therefore not weight normalized. Further details of this procedure are described elsewhere (Gooding and LeBlanc 2001).

**In vitro *effects of TBT on microsomal ATAT activity.*** The gonad-viscera complex was isolated from newly euthanized untreated snails and combined into 1-g samples (approximately 20–30 snails per sample). Tissue samples were homogenized in 100 mM potassium phosphate buffer (pH 7.4), 100 mM KCl, 1.0 mM EDTA, 1.0 mM diothiotreitol, 0.1 mM phenanthroline, 0.5 mg/mL trypsin inhibitor (Sigma, St. Louis, MO) (Lee et al. 1982). Microsomes were prepared by differential centrifugation (van der Hoeven and Coon 1974). The microsomal

**Table 1.** Testosterone levels measured in female snails after 3-month exposure to TBT.[a]

| TBT (ng/L) | No.[b] | Testosterone (pg/mg) | | | Free testosterone (%) |
|---|---|---|---|---|---|
| | | Free | Ester | Total | |
| Control | 11 | 1.8 (0.2) | 25 (2) | 26 (3) | 7.2 (0.6) |
| 0.10 | 8 | 2.1 (0.5) | 26 (5) | 29 (5) | 8.5 (1.6) |
| 1.0 | 10 | 2.3 (0.4) | 27 (4) | 30 (4) | 8.2 (1.1) |
| 10 | 11 | 3.1 (0.3)* | 22 (3) | 25 (3) | 15 (2)* |

[a]Values are presented as means with standard errors in parentheses. [b]Number of snails analyzed from each treatment.
*Significant difference ($p$ < 0.05) from the control.

pellet was resuspended in buffer [100 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, and 20% glycerol] and microsomal protein concentrations were determined (Bradford 1976) using commercially available reagents (Bio-Rad, Hercules, CA) and bovine serum albumin (Sigma) as a standard. Microsomes were stored at –80°C until assays were performed.

Microsomal ATAT activity was measured using microsomes (250 µg of protein), 150 µM palmitoyl CoA (Sigma), 150 µM [$^{14}$C]testosterone (100,000 dpm), and 0.1 M potassium phosphate buffer (pH 7.6). Total assay volume was 500 µL. TBT (0.01–10 µg/L) was included in the assays when evaluating the inhibition of ATAT by TBT. Reactions were allowed to run for 0, 15, and 30 min at 35°C. These conditions were shown in preliminary experiments to produce a quantifiable amount of product at a constant rate of production. Samples were then extracted with 3.0 mL ethyl acetate, resolved by TLC, and quantified by autoradiography as described for [$^{14}$C]testosterone–fatty acid esterification.

***Total tin analyses.*** Mud snails were collected from either Bald Head Island Creek or Bald Head Island Marina and tissues were stored at –80°C until analyzed. Snails from the creek were pooled for total tin determination (five snails per sample). Snails from the marina were typically larger and were either pooled (three snails per sample) or analyzed individually. Wet tissue samples were heated in 5.0 mL concentrated OmniTrace (EM Science, Gibbstown, NJ) nitric acid in a microwave digester for 10 min. After cooling to room temperature, samples were brought up to a 100-mL volume with distilled, deionized water. Instrumental analysis for tin was conducted on 50 mL of sample with Indium (100 ppb) as an internal standard on a Perkin Elmer ELAN 6000 Inductively Coupled Plasma Mass Spectrometer (Perkin Elmer, Downers Grove, IL).

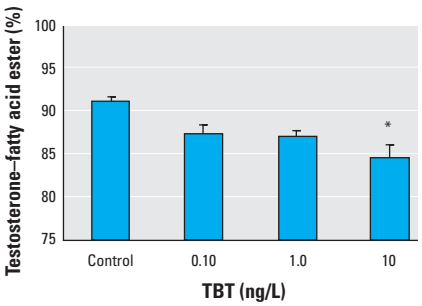


**Figure 2.** Percentage of administered [$^{14}$C]testosterone converted to and retained in snails as fatty acid ester after exposure to TBT for 3 months. Error bars represent SEs ($n$ = 8 snails).
*Significant difference from the controls at $p \leq 0.05$.

***Statistics.*** A contingency table followed by a Chi-square test was used to test the significance of imposex induction ($p$ = 0.05) when compared with the control. Statistical significance when comparing multiple treatment groups to a control group was determined by ANOVA and Dunnett's multiple comparison test with data containing homogeneous variances. Data were analyzed by Kruskal-Wallis nonparametric ANOVA and distribution free multiple comparisons when variances were not homogeneous. Homogeneity of variance was assessed by Bartlett's test. Statistical analyses were performed using JMP statistical software (SAS Institute, Cary, NC).

## Results

***Induction of imposex.*** Exposure of mud snails to concentrations of TBT for 6 months marginally induced imposex at 0.10 ng/L and significantly ($p$ = 0.05) induced imposex at 1.0 ng/L and 10 ng/L TBT (Figure 1). Among the several TBT exposures (3 and 6 months) performed as part of this study, the incidence of imposex in the controls was typically less than 5% and the maximum imposex incidence attained by TBT exposure ranged from 25 to 35%.

***Testosterone levels after TBT exposure.*** Testosterone levels were measured in snails after a 3-month exposure to concentrations of TBT in an effort to substantiate previous observations (Bettin et al. 1996; Oehlmann et al. 1993; Spooner et al. 1991) that TBT elevates testosterone in snails that are susceptible to imposex. Total testosterone levels were not elevated after exposure to TBT (Table 1). However, the concentration and percentage of testosterone that was available in the free form was increased with increasing exposure level. These observations were consistent with our hypothesis that TBT interferes with the storage of testosterone as fatty acid esters.

***Fatty acid esterification of testosterone after TBT exposure.*** Snails were exposed to concentrations of TBT for 3 months and then evaluated for their ability to convert [$^{14}$C]testosterone to [$^{14}$C]testosterone–fatty acid ester. The accumulation of [$^{14}$C]testosterone–fatty acid ester decreased with increasing TBT exposure level, with a significant reduction in fatty acid ester evident at 10 ng/L TBT (Figure 2). Thus, TBT inhibits the ability of the snails to produce or retain testosterone as the fatty acid ester.

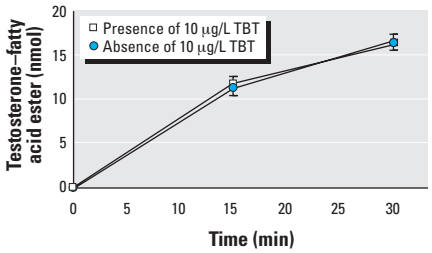


**Figure 3.** Microsomal ATAT activity in the presence and absence of 10 µg/L TBT. Results are presented as the mean and SE of three individual analyses.

***Direct inhibition of ATAT activity by TBT.*** Experiments were performed to determine whether TBT directly inhibited microsomal ATAT activity, the enzymatic activity responsible for the conversion of testosterone to testosterone–fatty acid ester. Gonadal-visceral microsomes were incubated with concentrations of TBT as high as 10 µg/L and evaluated for ATAT activity. The rate of conversion of [$^{14}$C]testosterone to the fatty acid ester was comparable among TBT-treated and untreated microsomes (Figure 3). These results imply that TBT does not increase free testosterone levels by directly inhibiting the ATAT enzyme.

**In vivo *suppression of ATAT by TBT.*** Experiments were conducted to determine whether TBT might increase free testosterone levels by suppressing the level of expression of the ATAT enzyme. This possibility was evaluated indirectly by exposing snails to 10 ng/L TBT for 3 months, preparing microsomes from the TBT and control treated snails, then measuring ATAT activity in these microsomes in the presence of necessary cofactors (testosterone, palmitoyl CoA). Thirty percent of the snails exposed to TBT in this experiment developed imposex. Therefore, gonadal-viscera microsomes from imposex and nonimposex snails were separately prepared and assayed. Microsomal ATAT activity was comparable among controls and both TBT treatment groups (Figure 4). Thus, TBT does not suppress the level of expression of ATAT in mud snails coincident with imposex.

***Evaluation of a tin-affected population.*** Snails from a high-tin–affected area and low-tin–affected area were evaluated for imposex, free testosterone levels, and the percentage of testosterone present as the fatty acid ester to determine whether effects observed under controlled TBT exposures occurred in field-exposed populations. Total tin levels in tissue from snails collected from Bald Head Island Marina were approximately 9-fold higher than total tin levels in snails collected from Bald Head Island Creek (Figure 5A). All female snails collected from the marina had a discernible penis, whereas the incidence of imposex among snails collected from the creek was 5.5% (Figure 5B). Free testosterone levels were significantly ($p \leq 0.05$) higher in snails collected from the marina as compared with those collected from the creek (Figure 5C). Further, the percentage of testosterone retained as the fatty acid ester was significantly ($p \leq 0.05$) lower in snails collected from the

marina (Figure 5D). These results demonstrate that the effects of TBT on testosterone esterification documented under controlled laboratory conditions are consistent with effects observed among tin-exposed, imposex field populations.

## Discussion

We showed previously that the predominant testosterone biotransformation process in the mud snail is the conversion of testosterone to testosterone–fatty acid esters (Gooding and LeBlanc 2001). The purpose of the present study was to test the hypothesis that the increase in immunodetectable testosterone levels after TBT exposure reported by others and purported to be casually associated with imposex (Bettin et al. 1996; Oehlmann et al. 1993; Spooner et al. 1991) is caused by alterations in the ability of the snails to esterify testosterone. Results from the study confirmed that TBT exposure both induces imposex and elevates immunodetectable testosterone levels in the mud snail. In addition and consistent with our hypothesis, we also demonstrated that the increase in immunodetectable testosterone is not caused by an increase in total testosterone. Rather, TBT increased the proportion of total testosterone that was available in the free, immunodetectable form.

TBT has been reported to elevate testosterone levels in *N. lapillus* and *Hinia reticulata* (Bettin et al. 1996; Spooner et al. 1991). In all cases, testosterone levels were measured by immunoassay. Results from the present study indicate that these studies with other snail species that are susceptible to TBT-induced imposex may require reinterpretation. Immunochemical analyses do not detect fatty acid–esterified testosterone (Addo et al. 1989). Therefore, it is likely that previous reports of increased testosterone levels with TBT exposure actually reflect increases in the ratio of free to esterified testosterone.

A maximum of approximately 30% incidence of imposex could be attained under our TBT exposure scenario. This level of imposex is comparable with that attained in exposures to TBT in other laboratories (Oberdörster et al. 1998; Smith 1981). Yet, we (Gooding et al. 1999) (Figure 5B) and others (Axiak et al. 1995; Curtis and Barse 1990; Morcillo and Porte 1999; Smith 1981; Spence et al. 1990) have reported incidents of imposex greater than 95% in TBT-impacted marine locations. The reason why a maximum incidence of only about 30% imposex could be attained in our laboratory exposures is not known. Possible reasons include the duration of exposure and the life stage of the snails used in the experiments. Six months is the maximum duration that we have exposed snails to TBT. Perhaps exposure to the chemical for longer periods would produce a greater incidence of imposex. Furthermore, when collecting snails in the field, we preferentially selected the largest snails to increase the percentage of females in our samples. By selecting the largest snails, we may have preferentially selected older individuals. Mensink et al. (2002) demonstrated that adult female snails (*Buccinum undatum*) are insensitive to TBT-induced imposex, whereas this response was readily discernible in juveniles. Smith (1981) also noted an increased response to TBT from young female mud snails when compared with adult females.

The fatty acid esterification of testosterone by other species has been reported, though this typically represents a minor process in the biotransformation of testosterone (Addo et al. 1989; Borg et al. 1995; Kishimoto 1973). The fatty acid esterification of testosterone increases the lipophilicity of the testosterone molecule and presumably facilitates its storage in lipoidal matrices while reducing its bioactivity, bioavailability, and susceptibility to elimination processes (Borg et al. 1995). Indeed, we demonstrated previously that exogenously provided testosterone is not readily eliminated by the mud snail. Rather, the testosterone is converted to fatty acid esters and retained in the tissues of the organism (Gooding and LeBlanc 2001). We also observed that, regardless of the amount of testosterone administered to the snails, the amount of free testosterone measured in the tissues of the organism remains relatively constant, and all excess testosterone is converted to the fatty acid ester (Gooding MP, LeBlanc GA. Unpublished data) These observations suggest that the snails maintain physiologically appropriate levels of free testosterone by converting excess testosterone to the ester and perhaps drawing from this pool of esterified testosterone when additional free testosterone is required. TBT appears to alter this normal homeostatic relationship between free and esterified testosterone by increasing the portion of the total testosterone pool that exists in the free state.

In this study, TBT maximally reduced testosterone–fatty acid esters by only about 13% (Figure 2). However, a 13% reduction in esters present at a normal level of 25 pg/mg (Table 1, control) would add about 3 pg/mg to the free testosterone pool. This is consistent with the magnitude of increase in the free testosterone pool observed following TBT exposure (Table 1) and in snails collected from a high-tin–affected field site (Figure 5).

A role for testosterone as a hormone in molluscs has not been definitively established. Although we have readily induced penis development in female snails with TBT (Figure 1),

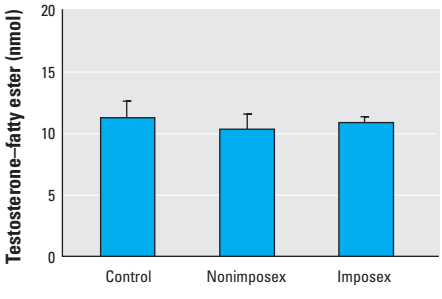


**Figure 4.** Testosterone–fatty acid ester production from microsomes prepared from control snails and snails exposed to 10 ng/L TBT for 3 months. Imposex and nonimposex females were analyzed separately. Error bars represent the SE from three analyses.

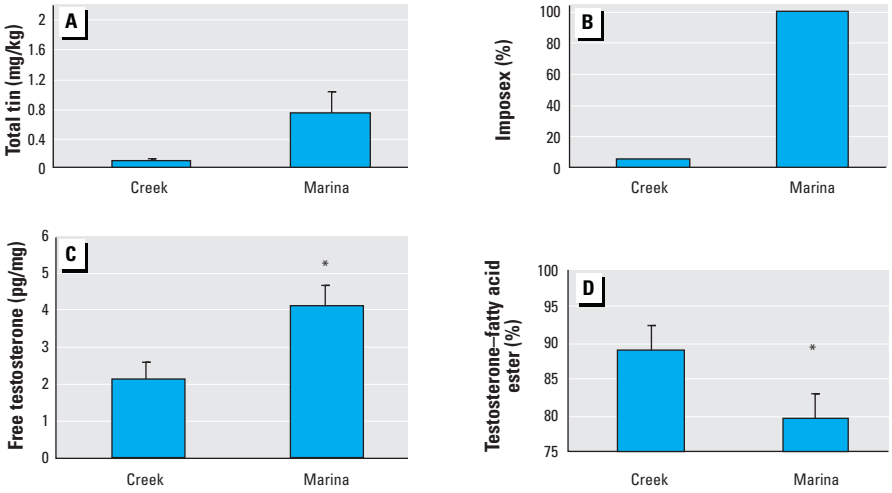


**Figure 5.** Analyses of snails collected from a high-tin–affected site (marina) and a low-tin–affected site (creek). (*A*) Tissue levels of total tin ($n = 6$, creek; $n = 13$, marina). (*B*) Incidence of imposex among the marina and creek populations. (*C*) Tissue levels of free testosterone (pg/mg). (*D*) Percentage of testosterone in tissues that was fatty acid esterified. (*B,C,D*), $n = 12$ . Error bars represent SE.
*Significant difference from the creek, $p \leq 0.05$.

attempts to induce penis development in snails in our laboratory with testosterone have been inconclusive. Recent studies conducted in our laboratory have indicated that snails may be susceptible to exogenous testosterone only during certain times within the reproductive cycle (Gooding MP, LeBlanc GA. Unpublished data). Nonetheless, administration of testosterone to various molluscan species has been shown to stimulate penis development and other masculinizing effects. A penis and/or vas deferens was induced in the marine snails *N. lapillus* and *H. reticulata* after administration of testosterone (Bettin et al. 1996; Spooner et al. 1991). In the castrated hermaphroditic slug *Euhadra pelionphala,* testosterone administration led to the development of a head wart, a secondary sex characteristic (Takeda 1980). Administration of testosterone increased spermatogenesis in the land snail (*Theba pisana*) (Sakr et al. 1992), the scallop (*Mizuhopecten yessoensis*) (Varaksina et al. 1992), and the snail (*Helix pomatia*) (Csaba and Bierbauer 1979). High-affinity testosterone-binding protein was characterized from the male reproductive organs of the octopus (*Octopus vulgaris*) (D'Aniello et al. 1996). We have recently commenced screening a mud snail cDNA library for the presence of an androgen receptor. Identification of a functional androgen receptor in this species would provide substantial evidence for a role for steroidal androgens in molluscan physiology.

The reduced esterification of testosterone by TBT may be indicative of imposex without being directly responsible for the phenomenon. For example, high-affinity 17β-estradiol binding proteins have been measured in mussels (Gagné et al. 2001), and precedent exists for estrogens being functional in molluscs (Di Cosmo et al. 2001; Takeda 1979). Perhaps the increase in free testosterone (the precursor to 17β-estradiol) resulting from TBT exposure increases synthesis of 17β-estradiol, with this hormone being the ultimate regulator of sex differentiation. Alternatively, the modulation of the free:esterified testosterone ratio by TBT may be reflective of similar modulation of another, yet unidentified hormone that is the ultimate regulator of male-sex differentiation. Under these scenarios, alterations in the testosterone free:ester ratio may serve as a biomarker of imposex, but may not be causally associated with the phenomenon. Further study is necessary to elucidate the precise role of testosterone in the sex differentiation of molluscs and TBT-induced imposex.

The precise mechanism by which TBT alters the free:ester testosterone ratio in the mud snail was not established in this study. Results did demonstrate that TBT neither directly inhibits the ATAT enzyme responsible for the fatty acid esterification of testosterone, nor does it suppress levels of the microsomal enzyme, as determined though activity assays following *in vivo* exposure to TBT. The possibility remains that prolonged exposure to TBT is required for either inhibition or suppression of the enzyme. Alternatively, TBT may affect some cofactor required for the esterification reaction, such as acyl CoA or fatty acid. Finally, TBT may induce the esterase responsible for releasing testosterone from the fatty acid moiety. Additional experiments are warranted to elucidate the mechanism by which TBT affects the levels of free testosterone.

The International Maritime Organization has called for a global prohibition on the use of organotin-based paints effective 1 January 2008 (International Maritime Organization 2001). Undoubtedly, this compound will be replaced by other chemicals with antifouling properties. Elucidation of the mechanism by which TBT alters hormones responsible for sex differentiation in molluscs resulting in imposex would help ensure that future TBT replacements do not share this notorious toxicological property.

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