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TESTOSTERONE METABOLISM IN THE ESTUARINE MYSID *NEOMYSIS INTEGER* (*CRUSTACEA; MYSIDACEA*) FOLLOWING TRIBUTYLTIN EXPOSURE

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Abstract—Current evidence suggests that the biocide tributyltin (TBT) causes the development of imposex, a state of pseudohermaphrodism in which females exhibit functional secondary male characteristics, by altering the biotransformation or elimination of testosterone. Imposex in gastropods following TBT exposure is the most complete example of the effects of an endocrine disrupter on marine invertebrates. Previous studies have demonstrated that the estuarine mysid *Neomysis integer* converts testosterone into multiple polar and nonpolar metabolites resulting from both phase I and phase II biotransformations. In this study, the effects of TBT chloride (TBTCl) on the phase I and II testosterone metabolism of *N. integer* were evaluated. The TBTCl was highly toxic to *N. integer* (96-h median lethal concentration [LC50] of 164 ng/L). To assess the effects on testosterone metabolism, mysids were exposed for 96 h to different concentrations of TBTCl (control, 10, 100, and 1,000 ng/L), and testosterone elimination as polar hydroxylated, nonpolar oxido-reduced, and glucose- and sulfate-conjugated metabolites was examined. The TBTCl differentially affected testosterone metabolism. The effect of TBTCl on phase I metabolism was unclear and has been shown to vary among species, likely depending on the inducibility or presence of certain P450 isozyme families. Reductase activity and metabolic androgenization were induced in the 10-ng/L treatment, whereas higher concentrations resulted in a reduction of sulfate conjugation. The exact mechanisms underlying TBT-induced imposex and alterations in the steroid metabolism need to be further elucidated.

Keywords—*Neomysis integer* Testosterone metabolism Tributyltin Endocrine disruption Biomarker

INTRODUCTION

Anthropogenic chemicals that can disrupt the hormonal systems (endocrine disrupters) of wildlife species have recently become a widely investigated and politically charged issue [1–3]. Invertebrates account for 95% of all animals [4], yet surprisingly little effort has been made to understand their value in signaling potential environmental endocrine disruption [5–7]. Presently, very few clear examples of endocrine disruption in invertebrates have been reported, perhaps because their hormonal systems have not been documented comprehensively [8].

Imposex in marine neogastropods following exposure to tributyltin (TBT), a marine biocide used in antifouling paints, is the most complete example of the effects of an endocrine disrupter on marine invertebrates [8]. This pseudohermaphroditic condition occurs at environmentally relevant concentrations [7] of TBT, which has been associated with population-level effects on marine neogastropods [9,10]. Although to our knowledge the underlying mechanism by which TBT causes imposex in gastropods has not been conclusively elucidated, the weight of evidence favors the aromatase-inhibition hypothesis [11]. This hypothesis states that higher levels of TBT in the presence of natural levels of testosterone probably inhibit competitively cytochrome P450–dependent aromatase (CYP19), thereby preventing the conversion of testosterone to 17β-estradiol [12–14]. This causes an increase in testosterone levels, leading to imposex induction. Alternatively, Ronis and Mason [15] suggested that TBT causes imposex in the periwinkle *Littorina littorea* by blocking phase II sulfate conjugation (and, hence, excretion) of testosterone and its potent metabolites (e.g., androstenedione, dihydroandrostenedione, dihydrotestosterone, and dihydrotestosterone-diols). However, this hypothesis could not be confirmed by Oberdörster et al. [16], who found no overall change in the elimination of testosterone as polar conjugates in TBT-exposed normal and imposex mud snails *Ilyanassa obsoleta*. Similarly, Gooding and LeBlanc [17] concluded that mud snails do not readily eliminate testosterone as polar derivatives. Finally, TBT can also interfere directly with the neurohormonal system of mollusks and, consequently, lead to changes in steroid titers only as a secondary effect [18,19]. Although TBT obviously interacts with some part of the endocrine system of mollusks [20], further investigations are needed to reveal the underlying mechanisms of imposex in these animals as well as to examine the interactions of this chemical with the hormone system of other invertebrates.

Most of the knowledge regarding crustacean endocrinology is derived from studies with larger decapods, such as crabs, lobsters, crayfish, and shrimp. Although some examples of laboratory experiments demonstrating the effects of endocrine disrupters in these animals have been reported, the conclusions are often ambiguous. Crustaceans may serve as good indicator species of endocrine disruption because of their economic importance, ecological significance, and extensive use as model invertebrates in laboratory toxicity testing [8]. In this context, we are investigating the potential of the estuarine mysid *Neomysis integer* as an indicator species for the potential effects

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of endocrine disrupters. Standard guidelines for conducting life-cycle toxicity tests with saltwater mysids have been developed using *Americamysis bahia* as a model species [21–23]. These standard methods, however, can be modified for testing with other mysid species [24]. The mysid *N. integer* dominates the hyperbenthic fauna of the low-salinity regions of western European estuaries [25,26], and it has been proposed as a European alternative to the standard test species *A. bahia* [27–31]. The work of Roast et al. [28–30] and of Verslycke and Janssen [31] demonstrates the successful use of this species for ecotoxicological work. Unfortunately, little information is available in the literature regarding the endocrinology of mysids.

Generally, most studies regarding the effects of endocrine disrupters in crustaceans have documented the interference of juvenile hormone analogues and other insecticides in the molting and metamorphosis of these animals [20]. A number of studies have been published concerning the steroid metabolism in crustaceans as a biomarker for the endocrine-disruptive effects of TBT. Several studies, for example, have investigated changes in the testosterone metabolism of daphnids following exposure to various xenobiotics, including TBT [7,32–37]. Other studies [38,39] have examined changes in the testosterone metabolism in daphnids (*Daphnia magna*) and the blue crab (*Callinectes sapidus*) following TBT exposure. In addition, we have recently reported on the steroid metabolism of *N. integer* [40].

To our knowledge, however, no studies regarding the effects of TBT on the steroid metabolism of mysids have been published. Hence, the purpose of this study was to examine the effects of TBT chloride (TBTCl) on the phase I and II testosterone metabolism of *N. integer*.

MATERIALS AND METHODS

Chemicals

Testosterone (4-androsten-17β-ol-3-one) and methyltestosterone (4-androsten-17α-methyl-17β-ol-3-one) were obtained from Sigma-Aldrich (Bornem, Belgium). Androstenedione (4-androsten-3,17-dione), dihydrotestosterone (5α-androstan-17β-ol-3-one), boldenone (1,4-androstadien-17β-ol-3-one), and the different testosterone metabolites (4-androsten-[2α-, 6α-, 6β-, 7α-, 11α-, 11β-, 15α-, 16α-, 16β-],17β-diol-3-one) were purchased from Steraloids (Newport, RI, USA). Tributyltin chloride (96% purity) was obtained from Janssen Chimica (Berchem, Belgium). Other solvents and reagents were analytical grade and purchased from Merck Eurolab (Leuven, Belgium).

Animal collection and maintenance

Initial *N. integer* populations were collected from the shore by handnet in the Galgenweel (a shallow, brackish water near Antwerp, Belgium). After a short acclimatization period, the organisms were transferred to 200-L glass aquaria. The culture medium was artificial seawater (Instant Ocean®; Aquarium Systems, Sarrebourg, France) diluted with aerated, deionized tap water to a final salinity of 5‰. A 14:10-h light:dark photoperiod was used during culturing, and water temperature was maintained at 15°C. Cultures were fed daily with 24- to 48-h-old *Artemia nauplii* ad libitum. Hatching of the *Artemia* cysts was performed in 1-L, cylinder-conical vessels under vigorous aeration and continuous illumination at 25°C.

TBTCl exposure

Juvenile mysids were randomly distributed in 1.5-L glass beakers (10 per concn., two replicates), with each containing 1 L of the required TBTCl concentration (control, 10, 100, 1,000, 10,000, or 100,000 ng/L for the toxicity experiment and control, 10, 100, or 1,000 ng/L for the testosterone metabolism experiment) in water with a salinity of 5‰ (diluted from artificial seawater [Instant Ocean]). The TBT was delivered to the exposure solutions in absolute ethanol. The ethanol concentration in the solvent control was 0.01%. Exposure temperature was 15°C, and test solutions were renewed after 48 h. Animals were fed twice daily with 24- to 48-h-old *Artemia nauplii* (~75 *Artemia*/mysid). Mortality was recorded daily.

TBTCl analysis

The pH of the medium was adjusted to 5.3 with a sodium acetate/acetic acid buffer before extraction. After ethylation in an aqueous solution containing 1% of tetraethylborate, organotins were extracted with hexane, separated with a Perkin-Elmer Autosystem (Norwalk, CT, USA) gas chromatograph (GC), and measured with a Perkin-Elmer Sciex Elan 5000 inductive-coupled plasma mass spectrometer (ICP-MS). Tripropyltin was used as internal standard. Details on the GC/ICP-MS operating conditions are given by De Smaele et al. [41].

Testosterone metabolism

Juvenile mysids were exposed to 10, 100, and 1,000 ng/L of TBTCl and to a solvent control as described above. Following the 96-h exposure period, mysids were individually placed into 5-ml glass tubes containing 2 ml of the same TBTCl concentration. Two micrograms of testosterone dissolved in 10 μl of methanol was added to the tubes, and a 6-h exposure period was applied. Exposure temperature was 15°C. Blanks were run to account for breakdown or microbial transformation of testosterone in the absence of mysids.

Neomysis integer were subsequently isolated from the exposure medium, dried on a paper towel, weighed on an analytical balance, shock-frozen in liquid nitrogen, and homogenized on ice in 100 μl of deionized water using a motor-driven Teflon® pestle. Methyltestosterone (50 ng in 50 μl of methanol) was always added before extraction as an internal standard. Testosterone metabolites were extracted from the homogenized organisms using 2 ml of ethyl acetate (2 × 1 ml) and phase-separated using centrifugation (5 min at 14,000 *g*). The two ethyl acetate fractions were pooled for analysis. Testosterone metabolites were extracted from the medium in the same way using 4 ml ethyl acetate (2 × 2 ml).

Following ethyl acetate extraction, the remaining polar phase II metabolites were hydrolyzed according to the method of Baldwin and LeBlanc [33]. In short, the assay medium was evaporated, and subsequently, the testosterone conjugates were hydrolyzed for β-glucose-, sulfate-, and α-glucose-conjugated metabolites and then extracted with 4 ml of ethyl acetate (2 × 2 ml).

Liquid chromatography with multiple mass spectrometry analysis of testosterone metabolites

The high-performance liquid chromatography apparatus was comprised of an Agilent 1100 series pump, autosampler, and vacuum degasser (Agilent, Palo Alto, CA, USA). Chromatographic separation was achieved using a Symmetry C_{18} column (5 μm, 150 × 2.1 mm; Waters, Milford, USA). The

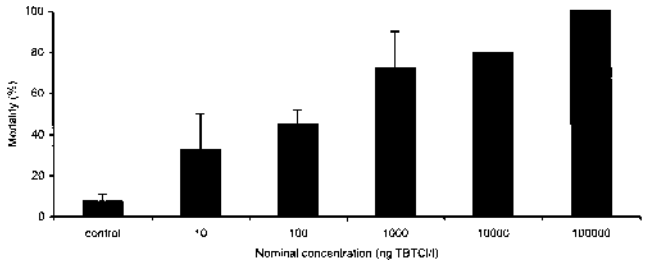

Fig. 1. Acute 96-h toxicity of tributyltin chloride (TBTCl) to the mysid *Neomysis integer*. Error bars represent standard deviation on the mean of two replicates ($n = 10$ in each replicate treatment).

flow rate was 0.3 ml min^{-1}. Analysis was carried out using an LCQ^{DECA} ion-trap mass analyzer (ThermoQuest, San Jose, CA, USA) with an atmospheric pressure chemical ionization interface and XCalibur 1.2. software (Batavia, NY, USA). The metabolites were detected in MS–MS–full scan positive-ion mode. The solvents for preparation of the mobile phase were high-performance liquid chromatography-grade obtained from Merck Eurolab. To separate and quantify the different compounds, samples were vacuum evaporated to dryness (Speedvac SC210A, Farmingdale, NY, USA) and reconstituted in 30 μl of MeOH and 90 μl of 0.02 M HCOOH. Sixty microliters were injected on column. A gradient elution was used (0.02 M HCOOH:MeOH, from 60:40 to 20:80 in 25 min, hold for 5 min). The different testosterone metabolites and endogenous steroids were identified on the basis of their relative retention times (calculated as a ratio of the retention time of testosterone) compared with the retention times of authentic standards. For more details, refer to Verslycke et al. [40] and De Wasch et al. [42]. All metabolite concentrations were normalized for the wet weight of the animals. The weights of the TBTCl-exposed mysids were not significantly different from the weights in the control (Dunnett's test, $p = 0.05$).

Statistics

The 96-h median lethal concentration (LC50) values were calculated using the moving-average method [43]. All data were checked for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene's test, respectively, with $\alpha = 0.05$. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test; Statistica™; StatSoft, Tulsa, OK, USA).

RESULTS

Acute toxicity of TBTCl to N. integer

Neomysis integer were exposed to 10, 100, 1,000, 10,000 and 100,000 ng/L of TBTCl for 96 h, and mortality was recorded (Fig. 1). Experimental TBTCl concentrations in freshly prepared test solutions and after 48 h were determined with GC-ICP/MS (Table 1). The experimental TBTCl concentrations in the freshly prepared solutions correlated well with the nominal values, except in the lower concentrations (10 and 100 ng/L). From the mortality data, a 96-h LC50 of 114 ng/L (95% confidence limits, 33–436 ng/L) could be calculated based on the nominal concentrations. Based on the measured average concentrations (average between concentrations in fresh solution and after 48 h), the 96-h LC50 was 164 ng/L (95% confidence limits, 52–373 ng/L).

Table 1. Aqueous tributyltin chloride (TBTCl) concentrations (ng/L) in the 96-h toxicity experiment with *Neomysis integer* as determined by gas chromatography/inductive coupled plasma mass spectrometer

Nominal TBTCl concentrations	Experimental TBTCl concentrations	
	Fresh solution	After 48 h
Control	8	9
10	32	28
100	150	86
1,000	1,050	1,210
10,000	8,540	2,630
100,000	ND[a]	ND

[a] ND = not determined.

Metabolic elimination of testosterone following TBTCl exposure

As previously described, *N. integer* produces a range of testosterone metabolites that can be divided into oxido-reduced/hydroxylated (phase I biotransformation) derivatives and conjugated (phase II biotransformation) derivatives [40]. Following 96-h exposure to TBTCl, the ability of the mysids to eliminate testosterone as various metabolic derivatives was evaluated (Table 2). The major metabolites detected in the medium were the nonpolar testosterone derivatives androstenedione and dihydrotestosterone. Dihydrotestosterone (not statistically significant) and androstenedione production was higher in the 10-ng/L treatment than in the control. Exposure of *N. integer* to 1000 ng/L resulted in a reduced production of nonpolar metabolites, but this was not statistically significant. Minor metabolites in the medium were the polar compounds boldenone, 6α-hydroxytestosterone, 11β-hydroxytestosterone, and 7α+15α-hydroxytestosterone. In some samples, 11α-hydroxytestosterone was also randomly detected in low concentrations. Mysids exposed to 100 and 1,000 ng/L produced lower amounts of polar monohydroxy metabolites compared to mysids in the control. This reduction was significant for the metabolite 7α+15α-hydroxytestosterone. Similar to the observed induction for the nonpolar metabolites, the concentration of some polar metabolites (boldenone and 11β-hydroxytestosterone) in the medium was significantly higher in the 10 ng/L treatment compared to the control.

The medium was also analyzed for phase II testosterone conjugates. The conjugation of testosterone and its metabolites to α-glucose was negligible in mysids. The elimination rates of β-glucose-conjugated, sulfate-conjugated, oxido-reduced, and hydroxylated testosterone and its derivatives were differentially affected by TBTCl exposure (Fig. 2). Sulfation and glycosylation were reduced after exposure to increasing TBTCl concentrations (significant reduction of sulfation in the 100- and 1000-ng/L treatments compared to the control).

Hydroxylation and conjugation of testosterone leads to inactivation and preferential elimination, whereas oxido-reduced metabolites are preferentially retained and many serve as androgens or androgen precursors [33]. Similar to Baldwin et al. [35], we used the ratio of the concentrations of the eliminated oxido-reduced products and the polar products (hydroxylated plus conjugated) to derive a metabolic androgenization ratio. This value has been used as a numerical interpretation for the total effect of a chemical on androgen metabolism in daphnids. Mysids exposed to 10 and 100 ng/L of TBTCl had higher metabolic androgenization ratios, but these were not signifi-

Table 2. Metabolic elimination of testosterone by *Neomysis integer* following 96-h exposure to tributyltin chloride (TBTCl)

Concn. (ng/L)	Polar metabolites[a]							Nonpolar metabolites[a]			
	Boldenone	2α-OH[b]	6α-OH	7α + 15α-OH	11α-OH	11β-OH	Total	AED[b]	DHT[b]	Total	TST[b]
Phase I											
Control (n = 10)	24.9 ± 12.3	—	18.8 ± 8.5	6.3 ± 2.3	—	20.1 ± 11.3	70.0 ± 28.4	2,005 ± 1,447	358 ± 453	2,363 ± 1,471	—
10 (n = 9)	40.01 ± 17.6[c]	—	19.1 ± 7.5	5.0 ± 2.1	—	29.1 ± 16.0	93.2 ± 27.5[c]	3,941 ± 1,912[d]	583 ± 482	4,524 ± 1,855[d]	—
100 (n = 8)	16.6 ± 5.2	—	13.4 ± 6.2	3.1 ± 1.0[d]	—	15.0 ± 3.4	48.1 ± 10.6	2,025 ± 1,119	293 ± 106	2,318 ± 1,152	—
1,000 (n = 8)	18.4 ± 6.2	—	12.3 ± 3.7	3.5 ± 1.6[d]	—	14.5 ± 6.2	48.6 ± 14.8	1,721 ± 1,011	189 ± 153	1,911 ± 1,049	—
Phase II											
Glycosylation											
Control	—	1.5 ± 1.1	—	—	2.9 ± 1.2	23.3 ± 15.5	27.7 ± 16.0	—	131 ± 44	131 ± 44	3,542 ± 1,610
10	—	1.2 ± 0.7	—	—	2.7 ± 1.8	25.0 ± 16.0	28.9 ± 17.8	—	190 ± 162	190 ± 162	3,440 ± 1,237
100	—	0.9 ± 0.4	—	—	4.0 ± 2.8	16.0 ± 8.9	20.8 ± 10.2	—	250 ± 110[c]	250 ± 110[c]	2,389 ± 1,161
1,000	—	0.8 ± 0.7	—	—	4.2 ± 1.4	18.0 ± 6.0	22.9 ± 6.4	—	151 ± 60	151 ± 60	3,103 ± 984
Sulfation											
Control	—	3.7 ± 3.0	—	3.0 ± 2.5	4.1 ± 2.3	34.0 ± 20.2	44.8 ± 23.6	—	278 ± 155	278 ± 155	5,319 ± 3,563
10	—	3.0 ± 0.5	—	2.7 ± 1.3	3.7 ± 1.4	36.2 ± 16.9	45.7 ± 18.3	—	268 ± 148	268 ± 148	5,018 ± 1,692
100	—	3.0 ± 1.1	—	1.8 ± 1.3	1.8 ± 0.8[d]	17.3 ± 10.6[c]	23.9 ± 11.9[c]	—	246 ± 148	246 ± 148	2,432 ± 838[c]
1,000	—	2.3 ± 0.9	—	1.1 ± 0.3[c]	3.0 ± 1.5	17.1 ± 6.0[c]	22.9 ± 6.8[c]	—	155 ± 67	155 ± 67	2,826 ± 991[c]

[a] Values obtained from liquid chromatography with mass spectrometry analysis. Data are presented as mean ± standard deviation in ng/g wet weights.
[b] OH = hydroxytestosterone; AED = androstenedione; DHT = dihydrotestosterone; TST = testosterone.
[c] Significantly higher/lower than the control at p = 0.05.
[d] Significantly higher/lower than the control at p = 0.01.

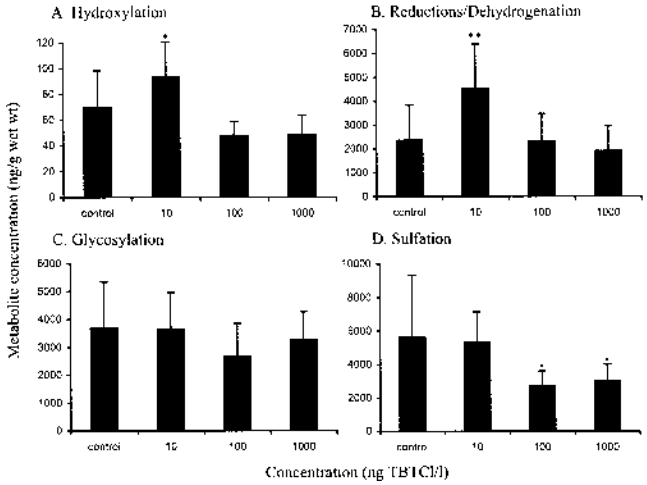

Fig. 2. Metabolic elimination of testosterone by *Neomysis integer* following 96-h exposure to tributyltin chloride (TBTCl) as hydroxylated (**A**), oxido-reduced (**B**), glucose-conjugated (**C**), and sulfate-conjugated (**D**) testosterone derivatives. Error bars represent standard deviation on the mean. Significance testing was performed by analysis of variance followed by post-hoc Dunnett's test (*$p < 0.05$, **$p < 0.01$).

cantly different from that of the control (Dunnett's test, $p = 0.1$ for 10 ng/L and 0.4 for 100 ng/L) (Fig. 3).

DISCUSSION

Acute toxicity of TBTCl to N. integer

Literature regarding toxicity testing with the standard mysid species *A. bahia* demonstrates the high sensitivity of mysids, in many cases at levels that are likely to occur in the environment [24,44,45]. The acute LC50 for juvenile *N. integer* of 164 ng/L of TBTCl found in this study is lower than that reported by Goodman et al. [46] for *A. bahia* (1,100 ng/L of TBT). Davidson et al. [47] and Valkirs et al. [48] also observed acute TBT toxicity within the same range (300–420 ng/L) for mysid shrimp (for review, see Fent [49]). Tributyltin compounds thus are highly toxic to juvenile mysid shrimp. It can be speculated from these results that coastal TBT contamination, which can still reach concentrations of 200 ng/L despite restrictive regulations [50], may be a potential threat to resident mysid populations.

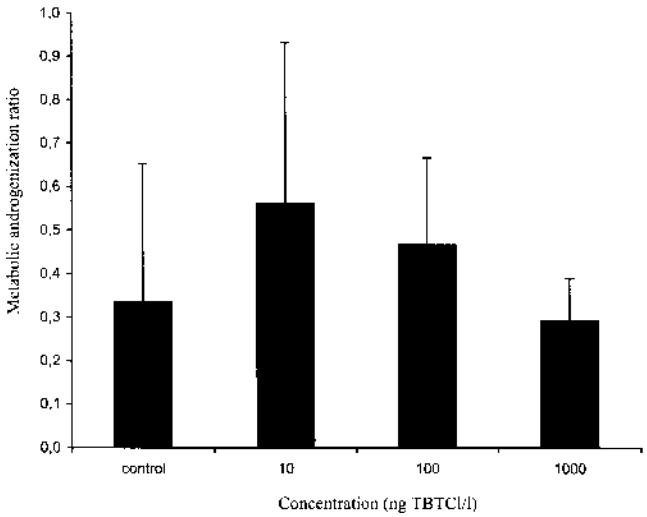

Fig. 3. Metabolic androgenization of tributyltin chloride (TBTCl)-exposed mysids, calculated as the ratio of oxido-reduced to glucosylated/sulfated/hydroxylated metabolites of testosterone [37]. Error bars represent standard deviation on the mean.

Table 3. Retention of testosterone (TST) and androstenedione (AED) by *Neomysis integer* following 96-h exposure to tributyltin chloride (TBTCl)

Concn. of TBTCl (ng/L)	Androgens measured in organism (ng/g wet wt)[a]	
	TST	AED
Control ($n = 10$)	285.7 ± 113.9	95.6 ± 58.5
10 ($n = 9$)	259.5 ± 114.8	154.9 ± 79.7[b]
100 ($n = 8$)	157.8 ± 72.1[c]	95.7 ± 69.1
1,000 ($n = 8$)	148.2 ± 43.8[c]	55.1 ± 28.0

[a] Data are presented as mean ± standard deviation.
[b] Significantly lower than the control at $p = 0.05$.
[c] Significantly higher than the control at $p = 0.01$.

Metabolic elimination of testosterone

Exposure to TBTCl differentially altered metabolic elimination of testosterone as phase I metabolites. An induction of both nonpolar and polar metabolites was observed in the lowest exposure (10 ng/L), but higher concentrations resulted in either no effect or a lower elimination rate.

The metabolism of boldenone (1,4-androstadiene-17β-ol-3-one) is poorly understood, but boldenone probably requires testosterone or androstenedione as a precursor [40]. Therefore, changes in boldenone production may reflect changes in the availability of these substrates. The production of both boldenone and androstenedione were significantly induced in the 10 ng/L of TBTCl treatment. Interestingly, we also observed a similar pattern for androstenedione body-burdens in the mysids exposed during this experiment (Table 3). Elimination of the nonpolar metabolite dihydrotestosterone was affected in the same way. Oberdörster et al. [16] described a similar maximum in the production of androstenedione at a concentration of 10 ng/L of TBTCl in the mud snail *Ilyanassa obselata*, which was lowered at the highest tested concentrations (20 and 200 ng/L).

None of the detected hydroxytestosterone metabolites were induced by TBTCl. It has been demonstrated that TBTCl induces the expression of CYP3A-like proteins in crab hepatopancreas [38]. Similarly, LeBlanc and McLachlan [51] observed an increased production of the CYP3A-dependent metabolite 6β-hydroxytestosterone in *D. magna* exposed to TBTCl. Other studies were unable to demonstrate changes in the phase I metabolism of TBTCl-exposed organisms [39]. The CYP2 and CYP3 families are largely responsible for hydroxylation of hormones and drugs. We have previously demonstrated the ability of *N. integer* to metabolize testosterone to different monohydroxymetabolites, such as 2α-, 6α-, 11α-, 11β-, 7α-, 16α-, and 15α-hydroxytestosterone [40]. All these metabolites are linked to the CYP2 family. No monohydroxymetabolites of testosterone linked to the CYP3A family (6β-, 2β-, 15β-, and 18β-hydroxytestosterone) were ever identified in our studies, which explains the present results. Apparently, TBTCl upregulates the expression of certain P450 isozyme families that are not ubiquitous in all invertebrates, resulting in a variable response among species (for a review of crustacean P450, see James and Boyle [52]).

Except for a small induction in the amount of glucose-conjugated dihydrotestosterone, glucose conjugation of testosterone metabolites in *N. integer* was unaffected by TBTCl

exposure. Elimination of testosterone as a glucose conjugate was not significantly affected by TBTCl but was lower in the two highest treatments compared to the control. Sulfate conjugation was significantly lowered, in a concentration-dependent manner, for both polar and nonpolar metabolites and testosterone. Ronis and Mason [15] hypothesized that TBT inhibits sulfur conjugation of testosterone as well as its metabolites and their excretion, resulting in a build-up of pharmacologically active androgens in the tissues. Our study corroborates, in part, the findings of Ronis and Mason, because the major biochemical targets of TBT appeared to be sulfate conjugation. However, we could not demonstrate that the buildup of active androgens in whole-body homogenates of the exposed mysids coincided with a reduced sulfation in the 100 and 1,000 ng/L of TBTCl treatments (Table 3). On the contrary, testosterone concentrations (as derived from whole-body concentrations) were significantly lower at the two highest test concentrations. This could not be related, however, to an induced metabolic elimination of testosterone metabolites in the medium. These lower testosterone concentrations may have resulted from a reduced uptake of testosterone, although Ronis and Mason [15] found no indication of changes in testosterone uptake. Similarly, we found no differences in testosterone concentrations in the medium, indicating that testosterone uptake from the medium was similar in all treatments (data not shown). Considering that conjugation of testosterone decreased instead of increased at higher TBT concentrations, the processes behind the observed decline in endogenous testosterone remain unclear. Hypothetically, testosterone could have been partly eliminated as metabolites that were not identified by the described liquid chromatography with mass spectrometry method (extracts were only scanned for the metabolites mentioned in *Materials and Methods*). These unidentified metabolites could, for instance, be androstanediols or androstenediols as observed in testosterone elimination experiments with the daphnid *D. magna* and the mollusks *I. obselata* and *L. littorea* [15,16,51].

The overall effect of TBTCl on the metabolic elimination of testosterone can be summarized by the ratio of the eliminated oxido-reduced products and the polar products (hydroxylated plus conjugated), that is, by the metabolic androgenization ratio (Fig. 3). Exposure of *N. integer* for 96 h to 10 and 100 ng/L of TBTCl resulted in an increased metabolic androgenization ratio (although not statistically significant from that of the control). This effect can be attributed to an induction of the reductase activity, resulting in higher productions of the nonpolar metabolites androstenedione and dihydrotestosterone in the 10-ng/L treatment. This induction corresponds with an increased buildup of the pharmacologically active androgen androstenedione in the tissues of *N. integer* (Table 3). The 1,000-ng/L treatment had no effect on the metabolic androgenization ratio, although a significant decrease in sulfate conjugation was observed, indicating that this mechanism might be important in explaining imposex phenomena. The effect of TBTCl on phase I metabolism was unclear and has been shown to vary among species, probably depending on the inducibility of certain P450 isozyme families. As proposed by LeBlanc and McLachlan [51], the differential effect on phase II conjugation may be related to the position in the cell of the involved enzymes. Both testosterone hydroxylase and glycosyltransferase enzymes are in the endoplasmic reticulum, whereas sulfotransferase is a water-soluble protein in the cytosol. Future research should focus on the fundamental understanding of the mechanism behind TBT toxicity and the cellular effects of this compound on the enzymes involved in steroid regulation.

In conclusion, TBT is highly toxic to mysids, and at present environmental concentrations, acute or chronic toxic effects could result in mortality and a decline in coastal or estuarine mysid populations. The sublethal effects of TBT on steroid metabolism remain unclear. Although we could demonstrate significant alterations in the testosterone metabolism (especially reductase induction and reduction in sulfate conjugation) of *N. integer* following acute exposure to sublethal concentrations of TBTCl, the mechanisms involved need further elucidation.

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