Bull. Environ. Contam. Toxicol. (2005) 75:1163–1167

DOI: 10.1007/s00128-005-0871-0

Bulletin of Environmental Contamination and Toxicology

Effect of Tributyltin at Environmentally Relevant Doses on Levels of Sex Hormones in Female Clams *Meretrix meretrix*

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Received: 23 February 2005/Accepted: 5 October 2005

Tributyltin (TBT) is an effective biocide that enters the aquatic environment mainly from its employment in antifouling paints on vessels, but also via discharge of wastewaters and dumping sewage sludge. It was reported that the levels of TBT in water of the coastal environments of China was below 0.5 ng/L (the detection limit) to hundreds ng/L as Sn (Jiang et al. 2001). TBT has been shown to be very toxic to aquatic life, particularly to marine mollusks such as oysters (Alzieu and Heral 1984). Potential reproductive impairment has been reported at a concentration of TBT in water as low as l ng/L as Sn, viz. the induction of imposex, male characteristics on females, in dogwhelk (*Nucella lapillus*) (Gibbs et al. 1988). Despite the fact that molluscs, particularly gastropods, are known to be very sensitive to low concentrations of TBT, limited information concerning the mechanism of action of organotin compounds is available. Increases in testosterone titres or imbalance in the androgen:estrogen ratio have been described for several gastropod species after exposure to TBT and these findings have been associated with the phenomenon of imposex (Spooner et al. 1991; Schulte-Oehlmann et al. 1995; Bettin et al. 1996). However, there is a paucity of data on its effects on levels of sex hormones of clams (Morcillo et al. 1998; Morcillo and Porte 2000), particularly at environmentally relevant concentrations. The aim of the present work was therefore to determine the in vivo interaction of TBT at environmentally relevant concentrations with the levels of sex hormone of a clam (*Meretrix meretrix*), which is a commercially important (fisheries and aquaculture) mollusc species along the coast in China. The results will provide further information to assess impairment of TBT on reproductive potential of the clam.

MATERIALS AND METHODS

Tributyltin chloride (TBT) was obtained from Fluka AG, Switzerland, with a purity of greater than 97%. TBT was diluted in 98% ethanol to concentrations of 0.02-2mg/L as Sn. Radioactive steroids: [1,2,6,7-^{3}H]testosterone and [2,4,6,7-^{3}H]oestradiol were purchased from Amersham Biosciences Co, UK. All chemicals were of analytical grade and were obtained from commercial sources.

Clams (*Meretrix meretrix*) weighing 28~46g total weight, were captured from a coast in Xiamen city, Fujian province, China. Clams at first were maintained in tanks containing 60 L of aerated marine water, with natural photoperiod for 7 days.

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Clams were randomly selected, assuming equal percentage of males and females. 60 individuals for each exposure level were kept in one glass box with 1 individual/L filtered seawater, and were exposed via their water to TBT, at concentrations of 0.1, 1, 10 ng/L TBT as Sn in 98% ethanol respectively, at 13-15.5 ℃, salinity of 22-23. Clams were fed algae (*Chlorella* sp.) daily. The water containing different concentrations of the pollutant was exchanged by half every day. The control group received an equal volume of the solvent ethanol (50 μl/L). Individuals were taken after exposure for 2, 8 and 20days and transferred to clean water to recover for 7 or 20 days.

Females (yellow) were distinguished from males (white) by the color of the gonad. Whenever the color of the gonad was unclear, sex was determined by microscopic observation of a smear of gonadal tissue on a glass slide (400 magnification). Gonad tissues were frozen in liquid N_2 immediately after collection and stored at -80 ℃ for subsequent analyses as described below.

Gonad tissues were analyzed individually. Ovaries were cut into small pieces of 0.25 g wet weight for analysis. Steroid extraction was performed as described in Bettin et al. (1996), and assayed for testosterone and estradiol levels using a commercial radioimmunoassay (RIA) kit (Furui Biological Engineering Co, Beijing, China). The efficiency of the extraction procedure was checked by injecting clam with tritiated testosterone (650pg/g wet wt, 0.2 μCi) and tritiated oestradiol (60 pg/g wet wt, 0.02 μCi) and the recovery was 70±4% (n=4) for testosterone and 78±4% (n=4) for oestradiol. The limit of detection of the whole determination procedure was 0.2 ng/g wet weight for testosterone and 10 pg/g wet weight for oestradiol.

Results are reported as mean±SDE (standard error). The data were processed by parametric statistical analysis (ANOVA) using SPSS 10.0 software followed by the t-test and $P<0.05$ was accepted as significant.

RESULTS AND DISCUSSION

Mortality during the 40 day exposure period was <10% and no significant mortality differences were observed between the control and treated groups. Ethanol was used as the solvent in the present study. Control groups received an equal volume of the solvent. The result showed that the estradiol and testosterone content were not significantly affected by ethanol (Fig.1).

Fig.1 shows the effect of TBT on levels of sex hormones in the ovaries of *Meretrix meretrix*. A significant increase in testosterone levels was found in the clams exposed to 1 ng/L TBT for 8 days, and to the all concentrations of TBT for 20 days compared with the matched control. Significant increases were still observed after transfer to clean recovery tanks for 7 days, and 20 days in the clams exposed to 1 ng/L and 10 ng/L of TBT.

The estradiol levels were not significantly affected by TBT exposure. However, a significant increase in estradiol levels was observed in the clams exposed to 1 ng/L and 10 ng/L of TBT after transfer to clean recovery tanks for 20 days.

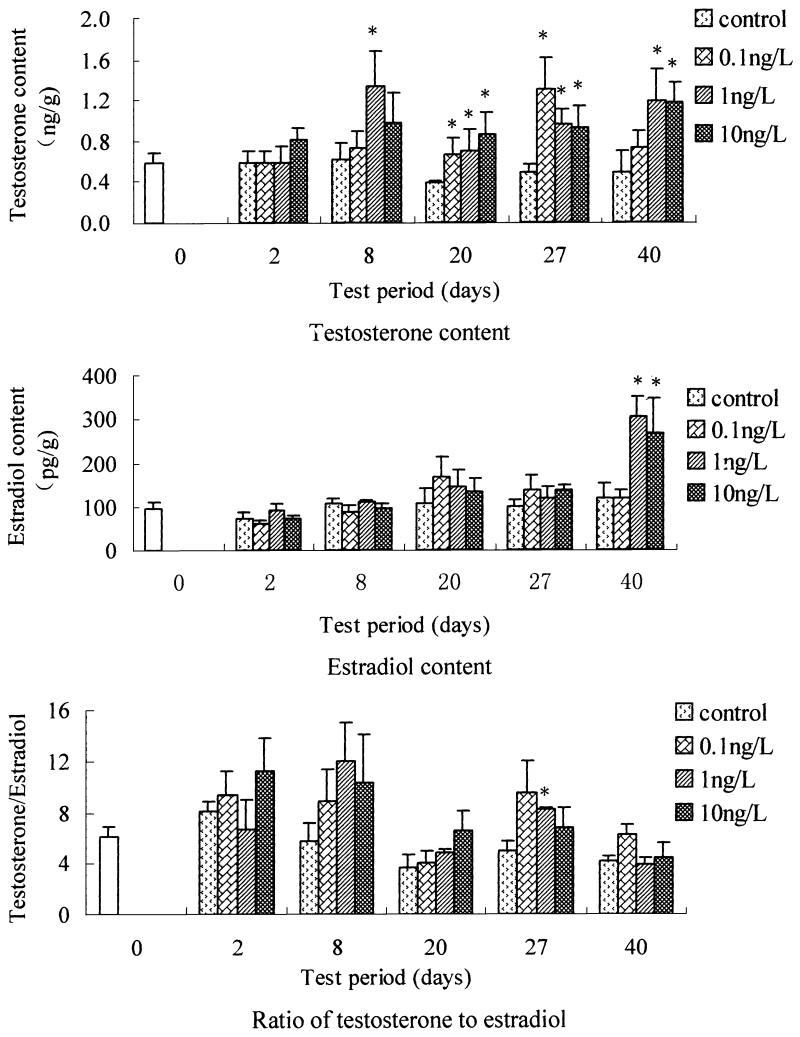

Figure 1 The effect of TBT exposure on testosterone content, estradiol content and the ratio of testosterone to estradiol in the ovaries of *Meretrix meretrix* TBT supply was removed on day 21. n=4-6 *, $P<0.5$; vs the control group.

Increases in the testosterone:estradiol ratio were observed in TBT-exposed clams, though there were not many significant differences among control and treated groups. Only a significant increase was observed in the clams exposed to 1 ng/L of TBT after transfer to clean recovery tanks for 7 days.

Exposure of the clam to different concentrations of TBT in the present study led to

significant increases in testosterone titres, which suggest the interaction of TBT with hormone metabolism. These findings were consistent with other studies that demonstrated increases in testosterone levels together with an increase in penis length in TBT-exposed gastropods (Spooner et al. 1991; Bettin et al. 1996; Morcillo et al. 1998). There is limited data available concerning the potential effect of TBT on estradiol levels, and conflicting results have been published. Namely, no significant changes in oestradiol titres were observed in TBT-exposed gastropods, apart from a transient and small decrease of estradiol in *Marisa cornuarietis* (Spooner et al. 1991; Schulte-Oehlman et al. 1995; Bettin et al. 1996). In the present study, the estradiol content was not affected by TBT exposure, but was significantly elevated after transfer to clean recovery tanks for 20 days, this phenomenon is difficult to explain. Up to the present, two hypotheses have been presented to explain the increase in testosterone titers with TBT: (1) the inhibition of the aromatase (CYP 19A1) enzyme which converts testosterone to estradiol (Spooner et al. 1991) and (2) interference with the metabolic elimination of testosterone (Ronis and Mason 1996). According to the aromatase inhibition hypothesis, TBT prevents the conversion of testosterone to 17β-estradiol. If the aromatase enzyme is the target of TBT, then a stoichiometric decrease in 17β-estradiol should occur commensurate with increases in testosterone (Gooding and LeBlanc 2001). In the present study, TBT-exposed individuals showed increases in testosterone titers with no corresponding decrease in 17β-estradiol; consistent with the studies of Spooner et al. (1991). This suggests that increases in testosterone might be due not to direct inhibition of aromatase, but to an indirect effect such as downregulation. Féral and LeGall (1983) demonstrated that TBT increases the neurosecreta of the pedal ganglia of female *Ocenebra erinacea*. However, significant elevation of estradiol content after transfer to clean recovery tanks for 20 days in the present study could result from relieving the inhibition of aromatase. This implied aromatase inhibition with TBT exposure. Effects of TBT exposure on the sex steroid hormones were continued after the clams were transferred to clean recovery tanks for 20 days. This might be due to TBT and its metabolites being poorly eliminated, such that they accumulated in the body of the clams. Morcillo and Porte (2000) suggested that metabolism of TBT were reduced in clams compared with other organisms.

Numerous investigations have proven that TBT is extremely toxic to aquatic organisms in general and to bivalve mollusks and gastropods in particular, for which the no effect levels are below 1 ng/L (Alzieu 2000). Zebrafish (*Danio rerio*) exposed for 70 days to as low as 0.1 ng /L of TBT showed a male biased population which produced a high incidence of sperm lacking flagella (McAllister and Kime 2003). However, there is limited information concerning effects in marine mollusks exposed to TBT below 1 ng/L. The testosterone content in the present work was induced after exposure to 0.1 ng /L of TBT as Sn for 20 days. The lowest observable effect concentration (LOEC; 0.1 ng /L) found in our study is below standard detection limits (>1 ng /L), and well below the EC permitted levels of receiving waters (2 ng /L; Environmental Agency 1998). This indicates that *Meretrix meretrix* would be a very sensitive species to TBT exposure. Though there is no penis in the clam, microstructure change of gonad tissues in the clam exposed to TBT should be further observed.

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