Influence of Short Term Exposure of TBT on the Male Reproductive Activity in Freshwater Prawn *Macrobrachium rosenbergii* (De Man)

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Abstract In the present study, the effect of tributyltin (TBT) on the histopathological and hormonal changes during spermatogenesis in freshwater prawn *Macrobrachium rosenbergii* was documented. Three experimental concentrations such as 10, 100 and 1,000 ng/L were selected and exposed to prawns for 45 days. After TBT exposure, the reproductive activities like sperm count and sperm length were decreased when compared with control. Further, abnormal structure of the seminiferous tubule, decrease in spermatozoa concentration, diminution of the seminiferous tubule membrane and the abundance of spermatocytes in the testis were noticed in treated prawns. Interestingly, radioimmunoassay clearly revealed the reduction of testosterone level in TBT exposed groups. Thus, TBT has considerably reduced the level of testosterone and caused the impairment of spermatogenesis in the freshwater male prawn *M. rosenbergii*.

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Freshwater ecosystem is under increasing threat due to rapidly expanding population and subsequent modernization process, which resulted in conspicuous exploitation of nature leading to pollution. The rivers are very vulnerable, since effluents from industries, domestic and farm open directly into them. During the past few decades, rising trends of population explosion, development of modern technology, industrialization and increase in the production and consumption of large variety of new synthetic chemicals are the causatives of enhanced release of pollutants in aquatic environment (Kharat et al. 2009). Accumulation of industrial effluents and agricultural runoff into water bodies has become a major concern (FAO 1986).

Organotin compounds, particularly tributyltin (TBT), have classically been reported to be strong endocrine disrupting compound (EDC), as they have been widely used as biocides in a variety of consumer and industrial products. TBT is a ubiquitous persistent xenobiotic that can be found in freshwater, estuarine and marine ecosystem (Fent 1996). Coexistence of TBT and triphenyltin (TPT) is still reported in aquatic environments and causes ecotoxicological threats to aquatic organisms because of its industrial applications (Zuo et al. 2012; Revathi et al. 2013a).

High concentration of TBT has been detected in bivalves and fishes collected from heavy organotin contaminated sites (Morcillo and Porte 1997). TBT causes morphological abnormalities and oogenesis in molluscs and crustaceans (Weis et al. 1987; Weis and Kim 1988, Revathi et al. 2013a). Though the mechanism of toxicity is unclear, triorganotins are thought to operate by inhibiting oxidative and photosynthetic phosphorylation processes essential to most of the organisms (Duncan 1980). The wide range and fundamental toxicity have raised concern over the effects of triorganotins on non-target organisms (Chiliamovitch and Kuhn 1977).

Histological analysis appears to be a very sensitive parameter and is crucial in determining cellular changes that may occur in target organs (Dutta 1996). Several studies have assessed the ability of endocrine disruptors to alter steroid hormone metabolism in invertebrates. Some studies have additionally assessed the impact of xenobiotics on endogenous steroid levels, which may in turn be an indication of altered steroid synthesis and metabolism (Revathi 2010).

Few studies have examined the effects at gametic levels, especially on sperm production (Mansueto et al. 2011; Revathi et al. 2013b). In our previous study, TBT showed significant inhibition of organogenesis as well as embryonic development in *Macrobrachium rosenbergii* (Revathi and Munuswamy 2010). The effect of TBT on the gametogenesis has been mostly studied in molluscs, however, only a limited number of research has been carried out on the impact of TBT in crustaceans peculiarly no much information is available on freshwater organisms like commercially important species *M. rosenbergii* (Revathi and Munuswamy 2010). Our previous study addressed the variation in ultrastructural, biochemical and sex hormone level in male prawn *M. rosenbergii* due to the long term exposure of TBT (Revathi et al. 2013b). However, the present investigation aimed to study the impact of TBT on spermatogenesis by evaluating cellular level variations in testis and quantification of testosterone in freshwater male prawn *M. rosenbergii.*

Materials and Methods

TBT was obtained from Himedia, India, with a purity of greater than 98 %. All other chemicals were of analytical grade and were obtained from commercial sources. Freshwater male prawn, *M. rosenbergii* were collected from the Aqua Nova hatchery in Kannathur near Chennai, South India. The collected prawns were brought to the laboratory in a plastic cover with habitat water. They were introduced into plastic tanks with sufficient aeration. The water was changed daily and they were fed ad libitum with commercial pelletized feed. They were maintained in the laboratory for 2–3 weeks for acclimatization.

The 5 month old prawns weighing 16 ± 2 g were selected and twenty prawns per group were exposed to TBT. The first group served as control (without any treatment). As ethanol is a solvent used to prepare the TBT (TBT chloride) solutions, the second group served as positive control that received 2 % ethanol treatment. Remaining three groups were individually exposed to environmentally realistic concentrations of TBT i.e., 10,100 and 1,000 ng/L using water as the medium (data not shown). Each group of prawns was maintained in the

individual plastic tanks containing 100 L of well aerated water and the water subjected to static renewal. Every day, the water was exchanged and the nominal concentrations of TBT were maintained in the respective experimental tanks. The experiment was performed for a period of 45 days. The water temperature was maintained at $18 \pm 2°C$. Three replicate tanks were maintained for each treatment group.

The water samples (250 mL) from each experimental tank were collected individually and stored at 4°C in the dark until laboratory analysis. The pH of the water samples was adjusted to 5 with 5 mL of sodium acetate buffer. To this, 100 µL of 2 % Sodium tetraethyl borate ($NaBEt_4$) was added and the mixture was stirred for 10 s. Then the mixture was allowed to stand for 10 min in the dark for reaction. Afterwards, 500 µL of isooctane was added to the mixture with vigorous magnetic stirring for 15 min. Then, Milli-Q water was added to obtain about a 3 cm high level in the narrow neck to facilitate decantation. From the mixture, 0.5 mL supernatant and 1 mL underlying aqueous phase were transferred into 2 mL effendorf tube and centrifuged for 5 min at 10,000 rpm at 4°C. Finally, the extracts were individually transferred into uncoated graphite tubes and immediately analyzed using graphite furnace atomic absorption spectrometry (GFAAS) at 286.3 nm for the quantification of TBT in the water samples (Michel and Averty 1991).

At the end of the experiment, the prawns were weighed and the gonads were removed and the wet weight of the gonads was recorded. The GSI and HSI were calculated following the procedure described by Zhang et al. (2007).

The sperm count of extruded spermatophoric mass was done following the direct cell count method (Tomar 1970).

Spermatophores were taken from the male prawn *M. rosenbergii* and it was homogenized with 1 % saline. From this solution one drop of the suspension was taken onto a slide and viewed under the Leica 2500 microscope. Randomly three samples of sperm were used for measuring their length using LAS software installed in Leica 2500 microscope (Germany).

The testes of male prawn in both control and treated groups were dissected and fixed in Bouin's fixative for histological studies. The tissues were dehydrated through a graded alcohol series and embedded in paraffin wax. Sections of 6–8 µm thickness were taken and stained with hematoxylin and eosin. The stained sections were mounted using DPX and photomicrographs were taken using Leica 2500 microscope (Germany).

The testes were dissected out from control and experimental prawns, pooled, shock frozen in liquid nitrogen and homogenized individually on ice in 100 µL deionized water using a motor driven Teflon pestle. Testosterone metabolites were extracted using 4 mL ethyl acetate (2×2 mL) and the organic phase was separated using

Table 1 Measurement of concentration of TBT in experimental tanks

Experimental groups	Measured concentration of TBT
Control	Nil
10	8.90 ± 0.72
100	84.6 ± 2.33
1,000	892.7 ± 5.67

Each value is a mean $\pm$ SD of three replicates

centrifugation at $5,000 \times g$ for 15 min at 4°C. The ethyl acetate fractions were pooled and evaporated under a stream of nitrogen.

The level of free immunoreactive testosterone was estimated using radioimmunoassay (RIA) according to the protocol of Oreczyk et al. (1974). The testosterone extracts were reconstituted separately in 100 μL of gelatin phosphate buffer solution (GPBS) (sodium phosphate buffer 0.1 M, pH 7.2, containing 0.15 M NaCl and 0.1 % gelatin) in RIA tubes. Appropriately diluted antiserum to testosterone (New England Nuclear Corp., Boston, MA) and 0.1 mL of [³H]-steroids without antiserum (to determine non-specific binding) were included in every assay. At the end of incubation, the unbound steroids were adsorbed on dextran coated charcoal to separate it from bound steroids. For that, 0.3 mL of dextran coated charcoal (0.1 % dextran T70 and 1 % charcoal in PSB) was added to each tube. Afterwards, the tubes were centrifuged at $3,000 \times g$ for 20 min at 4°C. The supernatant was poured carefully without disturbing the charcoal pellet into the vials containing 5 mL of scintillation fluid (0.5 % PPO, 0.04 % POPOP and 25 % methanol I toluene). The vials were kept shaking at room temperature to extract steroids into aqueous phase and steroid levels were estimated using a liquid scintillation counter (Beckman, USA).

Normality and homogeneity of the relative reproductive index, sperm count, sperm length and testosterone level was statistically analyzed by following one way analysis of variance (ANOVA) and Tukey- Dunnett test using SPSS 16.0 to determine the significant variations between the control and TBT treated groups.

Results

The male reproductive system was studied by observing the cellular level changes in testis of both control and treated groups. Further, the male reproductive activity was assessed based on the GSI and HSI level, sperm count, sperm length, cellular level changes and sex steroid levels in TBT treated and control prawns.
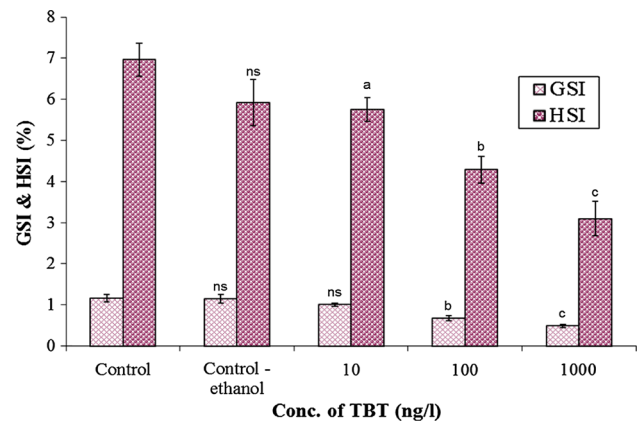

Fig. 1 Impact of TBT on the GSI and HSI in *Macrobrachium rosenbergii*. Each value is a mean $\pm$ SD of three replicates. *ns* non significant ($p > 0.05$); $^a p < 0.05$; $^b p < 0.01$; $^c p < 0.001$

Table 2 Effect of TBT on sperm count and sperm length in *Macrobrachium rosenbergii*

Experimental groups	Sperm count (millions/ spermatophoric mass)	Sperm length (μm)
Control	2.57 ± 0.28	8.11 ± 0.66
Control-ethanol	2.55 ± 0.21^{ns}	8.07 ± 0.76^{ns}
10	2.43 ± 0.11^{ns}	7.87 ± 0.54^{ns}
100	1.32 ± 0.13^{b}	6.75 ± 0.29^{ns}
1,000	0.33 ± 0.06^{c}	3.69 ± 0.09^{c}

Each value is a mean $\pm$ SD of three replicates

^{ns} non significant ($p > 0.05$)

^{a} $p < 0.05$

^{b} $p < 0.01$

^{c} $p < 0.001$

The concentration of TBT in experimental tanks was maintained by daily dosage after water exchange. At the end of the experiment, the concentration of TBT in experimental tanks was quantified using GFAAS and the values were 8.90, 84.6 and 892.7 ng/L TBT in 10, 100 and 1,000 ng/L of TBT treated tanks respectively (Table 1). One, one, two, three, four prawns died during the 45 days exposure period in the control, control-ethanol, 10,100 and 1,000 ng/L TBT treated groups respectively.

TBT had significantly reduced the GSI and HSI values in treated prawns compared to control (Fig. 1). In control, the GSI and HSI values recorded as 1.16 ± 0.09 % and 6.96 ± 0.40 % respectively. However, the GSI and HSI values were decreased to 0.49 ± 0.04 % and 3.1 ± 0.42 % at higher concentration of TBT (1,000 ng/L), respectively after 45 days of exposure. The GSI and HSI values statistically varied between control and TBT treated groups ($p < 0.05$ to $p < 0.001$).
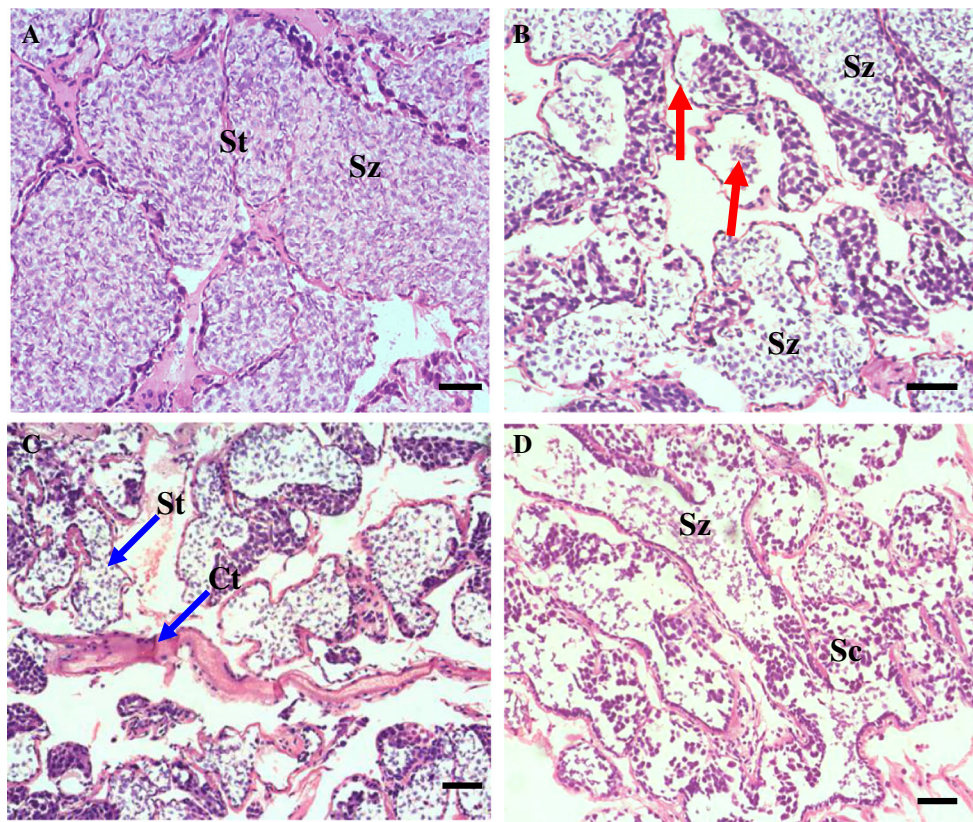
Fig. 2 a Control prawn (without treatment) testis showing fully mature spermatozoa (Sz) tightly packed with the lumen of seminiferous tubule (St). **b** At 10 ng/L TBT exposure, testis showing decrease in concentration of mature spermatozoa (Sz) in the seminiferous tubule and decrease in seminiferous tubule size (*up arrow*). **c** Testis showing disruption of seminiferous tubule architecture (*up arrow* St), increase in connective tissue level (*up arrow* Ct) at 100 ng/L TBT exposure. **d** At 1,000 ng/L TBT exposure, testis showing minimum concentration of mature spermatozoa (Sz), disruption of seminiferous tubule architecture, abundance of spermatocytes (Sc). Note the decrease in mature spermatozoa cells level in TBT treated groups compared to control. *Bar* 50 μm

Sperm count in the control prawn, was recorded as 2.57 ± 0.28 millions/spermatophoric mass (Table 2). In contrast, it drastically reduced to 2.43 ± 0.11 millions/spermatophoric mass, 1.32 ± 0.13 millions/spermatophoric mass and 0.33 ± 0.06 millions/spermatophoric mass, respectively in 10 mg/L, 100 mg/L and 1,000 ng/L TBT treated prawns. Statistical variations in sperm count between control and TBT treated groups was significant ($p < 0.05$ to $p < 0.001$).

Further, prawns exposed to TBT showed reduction in sperm length compared to control (Table 2). In control prawn, the sperm length was recorded as 8.11 ± 0.06 μm. However, in the treated groups, the sperm length was drastically reduced from 7.87 ± 0.54 μm (at 10 ng/L TBT) to 3.69 ± 0.09 μm (1,000 ng/L TBT). Sperm length between control and TBT treated prawns were statistically significant ($p < 0.05$ to $p < 0.001$).

In control prawn, mature spermatozoa were filled up in the lumen of the seminiferous tubules. However, in 10 ng/L TBT treatment, the testis showed decrease in seminiferous tubule diameter and decrease in the concentration of
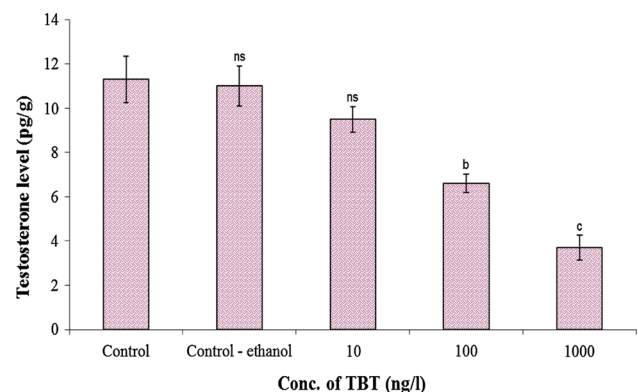

Fig. 3 Effect of TBT on the testosterone level in *M. rosenbergii*. Each value is a mean $\pm$ SD of three replicates. *ns* non significant ($p > 0.05$); [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$

spermatozoa. However, higher concentrations of TBT such as 100 and 1,000 ng/L resulted in disruption of seminiferous tubule architecture, increase in connective tissues, abundance of immature sperm cells like spermatogonia and spermatocytes (Fig. 2a–d).

The testosterone level in testis of control and treated groups was depicted in Fig. 3. The testosterone level in control prawn was measured as 11.3 ± 1.06 pg/g. However, there was a remarkable reduction in the testosterone level as the concentration of TBT increased from 10 to 1,000 ng/L. At higher concentration (1,000 ng/L), testosterone level decreased to 3.7 ± 0.57 pg/g in testis. Tukey-Dunnett test revealed that, the testosterone level in testis of TBT treated and control groups were statistically significant ($p < 0.05$ to $p < 0.001$).

Discussion

The present results inferred that, TBT possesses adverse effect on male reproductive system of *M. rosenbergii* as evidenced by reduced GSI, HSI values, retarding spermatogenesis, sperm count and sperm length. A decrease of 1.96-fold in GSI and 1.42-fold in HSI 2.42-fold sperm count and 1.0-fold sperm length was recorded at higher concentration (1,000 ng/L) of TBT. Due to the toxic effects of TBT the size of the gonads and hepatopancreas of the treated prawns was found to be decreased. Pesticides caused partial or total arrest of spermatogenesis may responsible for the reduction in sperm count and length in TBT exposed prawns as stated by Srivastava et al. (2008). Similar observation on the decrease of GSI in male prawn *M. rosenbergii* ascribable to TBT toxicity in the long term study has been reported (Revathi et al. 2013b). Accordingly, Revathi et al. (2013c) also described that TBT exposure led to the reduction in GSI and HSI values in mud crab *Scylla serrata*. Jegou (1992) found that dysfunction may lead to a reduction in sperm quality and possible infertility. Likewise Fent and Hunn (1995) suggested that TBT can also affect sperm count and male reproductive system in aquatic organisms. Accordingly, low levels of TBT exposure for 21 days decreased sperm count in guppies (*Poecilia reticulata*) (Haubruge et al. 2000). Nakayama et al. (2004) reported that TBT affects sexual behavior and reproduction in medaka (*Oryzias latipes*). Similarly, TBT even at environmentally realistic concentrations had also adversely affected the gametogenesis in cuvier *Sebastiscus marmoratus* (Zhang et al. 2007).

Testicular inflammation was documented as one of the common responses in aquatic animals exposed to environmental toxicants (Sokal et al. 1985; Ruby et al. 1986, 1987). The present results clearly documented the abnormal cellular architecture in testis and impairment of spermatogenesis in *M. rosenbergii* due to TBT toxicity. Overall, the results claim a dose dependent toxicity of TBT on the reproductive performance of *M. rosenbergii*. Zutshi (2005) observed appreciable reduction in size, with spermatids and sperms in degenerating condition and necrosis

of interstitial cells after fenthion treatment in fish, *Glossogobius giuris*. Zutshi and Murthy (2001) reported the extensive cytotoxic damage in the testis of *Glossogibius giuris* after fenthion exposure. Pesticides cause testicular atrophy such as disruption of seminiferous tubules and destroyed leydic cells that can arrest the spermatogenesis in fishes (Srivastava et al. 2008). Kumar et al. (2007) have also documented in vivo cytotoxic damage in testis of *Heteropneustes fossilis* exposed to anionic surfactant.

From the present study it is obvious that the TBT had significantly decreased testosterone level in testis of *M. rosenbergii*. Similar results was observed in male cover exposed to TBT (Zheng et al. 2005). Exposure of TPT led to a significant increase in fatty acid esterification of testosterone in *Paracentrotus lividus* (Janer et al. 2005). This is probably a mechanism to lower endogenous levels of testosterone by esterifying it with fatty acids. Increased sulfation may again be a mechanism to inhibit the biological activity of testosterone by decreasing its affinity to putative steroid receptors and facilitating its elimination (Lavado et al. 2006). The alterations of testosterone and energy metabolism are sensitive end points in mysids exposed to different xenobiotics (Verslycke et al. 2003a, b). Some other pesticides such as malathion, chlordane and lindane also caused a decrease in testosterone in aquatic organisms (Baldwin and LeBlanc 1994; Parks and LeBlanc 1996). In the above context, the present work clearly manifests that TBT had deleterious effects on spermatogenesis, reduction in testosterone level and impairment in the reproductive system of freshwater male prawn, *M. rosenbergii*.

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