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Influence of Tributyltin (TBT) on Level of Free Testosterone in Two Species of Neogastropods Found Along the Coast of Pakistan.

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Abstract:

Organotin compounds are used in antifouling paints and wood preservatives to prevent fouling of marine organisms on ships and other marine structures. The leaching of these compounds has caused imposex (pseudohermaphroditism) globally in females of over 170 species of prosobranch gastropods inhabiting areas close to marinas and harbours. The investigations of imposex induction pathway indicate that tributyltin (TBT) and triphenyltin (TPHT) produce this abnormality via endocrine dysfunction, interfering with the regulation of testosterone. The two gastropod species *Thais bufo* and *T. rudolphi* were analyzed from Manora Rocky Ledge, Clifton and Gadani Ship Breaking Yard. At Gadani Ship Breaking Yard the incidence of imposex was 100% while at the other two sites no imposex female was encountered. The testosterone levels were analyzed by using radio immunoassay (RIA). Higher levels of testosterone were recorded in imposex females than males at Gadani Ship Breaking Yard. However, from Clifton and Manora Rocky Ledge, elevated concentrations of testosterone were noted in males as compared to females. The relatively higher level of testosterone in imposex females from Gadani Ship Breaking Yard area indicates that TBT is an active biocidal agent released into marine environment during ship recycling process and is responsible for development of imposex and leading to elevated level of free testosterone in targeted species.

Keywords: Imposex; Neogastropods; Testosterone; Tributyltin

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1. Introduction:

Tributyltin (TBT) is a member of organotin (OTCs) family causing endocrine disruption due to its interference in normal hormonal regulation in a number of organisms [6]. TBT has been extensively used as biocidal agent in paints applied on ship hulls and on fishing nets to prevent fouling of organisms such as barnacles, seaweeds and gastropods [4, 18]. It is considered as the causative agent of imposex (superimposition of male sexual characters on females) in gastropods and endocrine disruption such as reduced fatty acid esterification leading to elevated levels of free testosterone in affected animals [13]. Furthermore, TBT also has an inhibitory effect on aromatase enzyme activity [14, 13], causes neurotoxicity [5] and disrupt functional role of retinoid X receptor (RXR) in imposex gastropods [20]. Due to potential role of TBT as endocrine disruptor which results in elevation of free testosterone levels in imposex females, the present study was carried out to compare free testosterone level in TBT sensitive neogastropod species collected from three different sites along the coast of Pakistan.

2. Material and Methods:

2.1 Sampling sites

The samples were collected from Gadani Ship Breaking Yard situated near the coastal village of Gadani located at (25° 7' N and 66°43' E) about 50 kilometer in the northwest of Karachi, Clifton (24°47' N 67° 05' E) is a popular residential and recreational area in Karachi which receives variety of pollutants in large amount from picnickers, domestic waste and oil spill etc. Manora Rocky Ledge (24° 48' N and 66° 58' E) is located in the southwest of Karachi facing the Arabian Sea. It is thousand meters long and two hundred and fifty meter wide and rich in fauna and flora (Figure no.1).

2.2 Sampling

The samples were randomly handpicked during the period September to November 2010. They were brought live to the laboratory, washed and cleaned thoroughly to remove epifauna and flora. They were kept live in aerated glass aquaria filled with sea water and narcotized for 30mins in 7% MgCl₂ solution and prior to the extraction of soft tissues. The soft tissues were extracted by cracking the shell with the hammer and then their sexes were identified and the tissues were stored at -20°C till further analysis.

2.3 Identification of male, female and Imposex

Males were identified by the presence of penis behind the right tentacle, prostate gland and vas deferens (VDS) and females by sperm ingesting gland and albumen gland. The imposex females were distinguished by the presence of VDS and penis (male sexual characteristics) in female gastropods [19].

2.4 Testosterone Analysis:

2.4.1 Extraction:

For extraction 10 mg tissues from viscera-gonad complex was homogenized in 400 µl of biophosphate buffer and extracted twice by adding 2.5 ml of diethyl ether. The mixture was vortexed and supernatants were collected in glass vials. Diethyl ether was evaporated by keeping the vials in a water bath at 40°C and reconstituted with 500 µl of assay buffer.

2.4.2 Estimation of testosterone:

Reconstituted samples (200 µl) were taken in disposable culture tubes, 100 µl bio-phosphate buffer, 100 µl of 3H labeled T as tracer (Amersham UK) and 100 µl T antibody (Guildhay UK) was added to these tubes. Standards were prepared, ranging from T 12.5 to 1600 pg/tube (Sigma), to these tubes same amount of tracer and antibody was also added. The assay tubes were incubated at 60°C and 30°C for 10 and 20 min respectively. Bound and unbound

fractions were separated by adding 500 μ l of dextran coated activated charcoal and incubating the tubes for 30min at 4°C. This mixture was separated by centrifugation in a refrigerated centrifuge at 4°C for 15min at 3000rpm. After centrifugation supernatant was decanted in scintillation vials containing scintillation cocktail (4 ml of 0.5 % permablend in toluene). Vials were vortexed for 1 to 2 min and kept overnight for stabilization. Radioactivity was measured as CPM/200 μ l in a scintillation counter. Testosterone concentration was calculated by Logit – log transformation. Sensitivity of testosterone assay was 0.0125 ng and the intra-assay coefficient of variation was <10%. The levels of testosterone in the media were expressed as pg or ng/ml.

3. Results

The one way analysis of variance (ANOVA) for testosterone level in between both species *T. bufo* and *T. rudolphi* revealed no significant difference at all three sites i.e. Gidani Ship Breaking Yard (F=0.41, $P \geq 0.05$), Clifton (F=1.14, $P \geq 0.05$) and Manora Rocky Ledge (F=3.57, $P \geq 0.05$).

The mean concentration of free testosterone in *T. bufo* from Gadani Ship Breaking Yard was (37.08 \pm 22.96; n=8 pg/mg) whereas; at Clifton and Manora Rocky Ledge it was (34.04 \pm 8.68 pg/mg; n=5) and (5.49 \pm 1.25 pg/mg; n=8) respectively. Similarly, in *T. rudolphi* estimated mean values of free testosterone were higher in Gadani Ship Breaking Yard samples (29.81 \pm 24.72 pg/mg; n=10), as compared to Clifton (26.51 \pm 12.56 pg/mg; n=4) and Monora Rocky Ledge (6.85 \pm 1.69 pg/mg; n=10).

Table1. Two-way analysis of variance (ANOVA) for effects of site and sex on level of testosterone in *T. bufo* and *T. rudolphi*

Species	source	d.f	F-ratio	P-value
<i>T. bufo</i>	site	2	12.98	0.001*
	sex	1	2.65	0.12
	site*sex	2	1.47	0.262
<i>T. rudolphi</i>	site	2	6.61	0.007*
	sex	1	0.31	0.587
	site*sex	2	2.59	0.103

(*) p values are significant (p <0.05).

Two way ANOVA was also applied to explain the difference in the level of free testosterone using site and sex as factor. In *T. rudolphi* no significant difference (F= 0.31, $P > 0.05$) was observed with respect to gender however, the level of free testosterone was statistically different (F= 6.61, $P < 0.05$) among sites. Additionally to understand the combined effect of both factors (gender and site) the interaction between gender and site for level of testosterone (F=2.59, $P > 0.05$) was tested, which clearly indicated that the difference in level of testosterone is due to the site while gender has no effective influence on level of testosterone. Likewise, in *T. bufo* no significant difference in the level of free testosterone was identified between genders (F=2.65, $P > 0.05$) although level of testosterone was significantly different among sites (F=12.98, $P < 0.05$). Furthermore, the interaction (F=1.47, $P > 0.05$) for site and sex in *T. bufo* has also indicated site based effect on testosterone level regardless of gender (Table no.1).

Estimated level of mean concentration of testosterone (pg/mg) (Figure no.2) was higher in imposex females of *T. bufo* (44.93 \pm 24.87 pg/mg) and *T. rudolphi* (44.63 \pm 28.52 pg/mg) than male *T. bufo* (24.00 \pm 14.01 pg/mg) and *T. rudolphi* (19.94 \pm 17.86 pg/mg) in sample analyzed from Gadani Ship Breaking Yard. Whereas, in Clifton samples the level of testosterone was lower in female *T. bufo* (29.74 \pm 13.97 pg/mg) and *T. rudolphi* (28.24 \pm 4.58 pg/mg) as compared to male *T. bufo* (36.90 \pm 4.71 pg/mg) and *T. rudolphi* (32.31 \pm 10.33 pg/mg). However, in Manora Rocky

Ledge samples the level of free testosterone in *T. bufo* (males: 5.77 ± 1.77 pg/mg; females: 5.21 ± 0.52 pg/mg) and in *T. rudolphi* (males: 7.68 ± 2.04 pg/mg; females: 6.28 ± 1.31 pg/mg) was comparatively very low.

4. Discussion:

In this study the level of free testosterone has been estimated in *T. bufo* and *T. rudolphi* collected from three different sites (Gadani Ship Breaking Yard, Clifton and Manora Rocky Ledge). Among these sites Gadani Ship Breaking Yard showed higher level of free testosterone as compared to other two sites, Clifton and Manora Rocky Ledge. The main reason for the elevated level of testosterone in specimen from Gadani Ship Breaking Yard seems to be the tributyltin used as main biocidal agent in antifouling paints used on ships which subsequently leached into the marine environment during ship breaking as reported from Fore River in South Portland [3, 13]. Moreover, presence of organotin at this site and its severe effect has also been revealed by incidence of 100% imposex in the population of female gastropods. Though, on morphological examination the specimens of *T. bufo* and *T. rudolphi* from Clifton and Manora Rocky Ledge showed no sign of imposex but higher level of free testosterone was detected in samples of Clifton as compared to Manora Rocky Ledge (clean site), suggesting that Clifton where different industrial and domestic effluents are being dumped, may receive other environmental pollutants such as bisphenol A, octylphenol [21], vinclozolin [12] and other endocrine disrupting metals [1] which could not induce imposex [7] but act as endocrine disruptor and affect the hormonal regulation. Furthermore, absence of imposex and lower level of testosterone in Manora Rocky Ledge samples as compared to two other sites indicated that this site is free of OTCs and other contaminants because it faces open sea and has no major industry and ship related activities such as recreational boating, commercial shipping and ship recycling in the area.

Result regarding the gender based analysis of free testosterone levels in *T. bufo* and *T. rudolphi* from Clifton and Manora Rocky Ledge showed higher level of free testosterone in males as compared to normal females in both species. However, at Gadani Ship Breaking Yard higher level of free testosterone were detected in imposex females than males. This clearly indicates the effect of TBT in testosterone regulation and imposex development [16]. Tributyltin causes the steroid imbalance and increase the level of free testosterone in female gastropods. This is further supported by the results of earlier investigations in which imposex developed after direct administration of testosterone in two gastropods, *Nucella lapillus* [2] and *Hinia reticulata* [5]. Elevated levels of free testosterone have been observed in imposex females of *Ilyanassa obsoleta* after exposure to TBT [13]. These findings therefore, indicate link between TBT related sexual abnormality and steroid regulation. However, the action mechanism of TBT is still unclear due to limited understanding of the endocrinology of gastropods [7].

Levels of steroid hormone have been estimated in different species of molluscs such as *I. obsoleta* [15], *Hexaplex trunculus*, *Bolinus brandaris* [16], *Mytilus edulis* [10], *Mya arenaria* [17], *Ruditapes decussates* [9] and in *Patinoplectin yessoensis* [11] and these studies suggested that the variation in levels of sex steroids not only related to reproductive cycle but also it is different among species [8]. However, in both *T. bufo* and *T. rudolphi*, no considerable differences in testosterone level has been observed in specimens examined from all three sites which demonstrate that influence of endocrine stressor on level of testosterone are similar in both species due to same physiological and environmental condition as they belong to same genus and live in the same habitat. However, further understanding of steroids hormone levels in relation to seasonal variation in gonadal development is required of two neogastropod species.

5. Conclusion:

Present work clearly indicates the endocrine disruptive effect of TBT on level of free testosterone in targeted gastropods. Further research work on action mechanism of TBT on level of free testosterone and other steroid hormone will provide better understanding of contamination dependent hormonal disorder in bioindicator gastropods. This study also reflects that the ship breaking yards are still highly contaminated with organotin compounds due to the recycling of old ships.

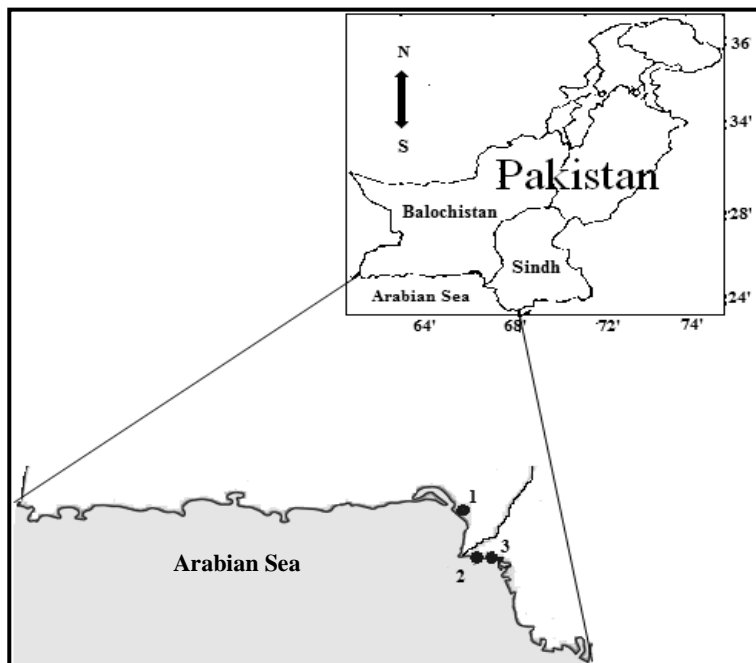


Figure1. Map showing the sampling sites. Gadani Ship Breaking Yard (1), Manora Rocky Ledge (2) and Clifton (3)

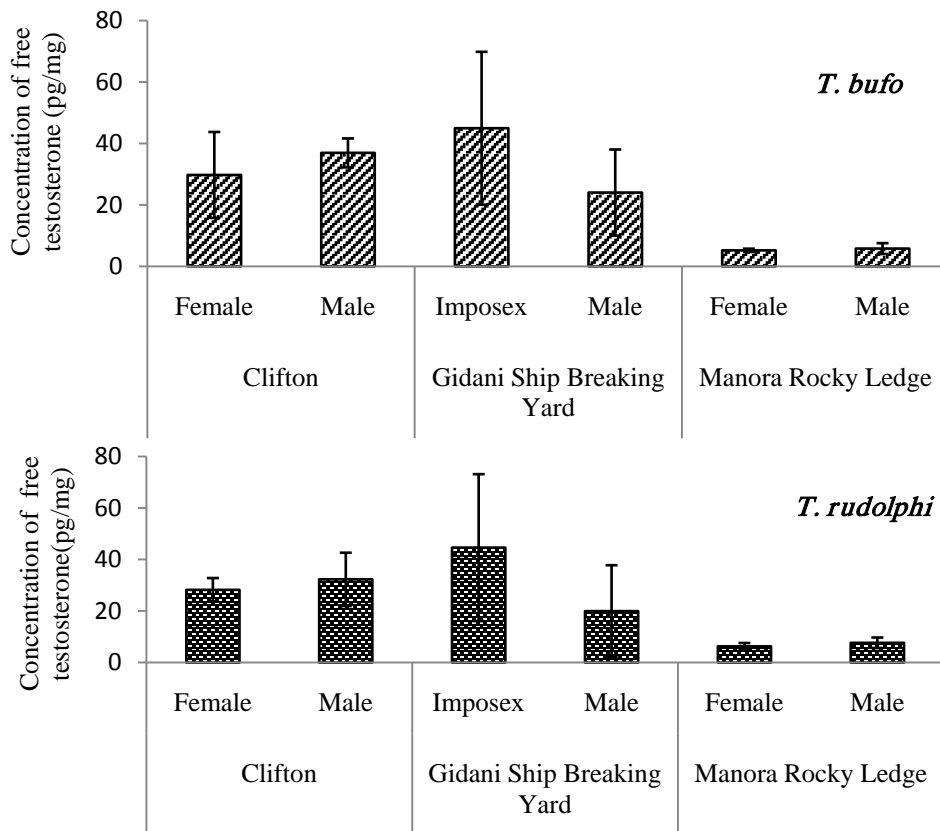


Figure2. Free testosterone in gonad and viscera complex of *T. bufo* and *T. rudolphi* studied from three different sites.

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