Aquatic Toxicology 162 (2015) 117–125

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Disruptions in aromatase expression in the brain, reproductive behavior, and secondary sexual characteristics in male guppies (*Poecilia reticulata*) induced by tributyltin

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ARTICLE INFO

Article history:
Received 21 November 2014
Received in revised form 16 March 2015
Accepted 18 March 2015
Available online 19 March 2015

Keywords:
Tributyltin
Secondary sexual characteristic
Reproductive behavior
Aromatase inhibitor
Sex hormone
Poecilia reticulata

ABSTRACT

Although bioaccumulation of tributyltin (TBT) in fish has been confirmed, information on possible effects of TBT on reproductive system of fish is still relatively scarce, particularly at environmentally relevant levels. To evaluate the adverse effects and intrinsic toxicological properties of TBT in male fish, we studied aromatase gene expression in the brain, sex steroid contents, primary and secondary sexual characteristics, and reproductive behavior in male guppies (*Poecilia reticulata*) exposed to tributyltin chloride at the nominal concentrations of 5, 50, and 500 ng/L for 28 days in a semi-static exposure system. Radioimmunoassay demonstrated that treatment with 50 ng/L TBT caused an increase in systemic levels of testosterone of male guppies. Gonopodial index, which showed a positive correlation with testosterone levels, was elevated in the 5 ng/L and 50 ng/L TBT treated groups. Real-time PCR revealed that TBT exposure had inhibiting effects on expression of two isoforms of guppy aromatase in the brain, and these changes at the molecular levels were associated with a disturbance of reproductive behavior of the individuals, as measured by decreases in frequencies of posturing, sigmoid display, and chase activities when males were paired with females. This study provides the first evidence that TBT can cause abnormalities of secondary sexual characteristics in teleosts and that suppression of reproductive behavior in teleosts by TBT is due to its endocrine–disrupting action as an aromatase inhibitor targeting the nervous system.

1. Introduction

Tributyltin (TBT) is a organotin chemical used for various industrial purposes such as slime control in paper mills, disinfection of circulating industrial cooling water, antifouling agent, and the preservation of wood. The use of TBT as biocides in antifouling boat paints leads to the contamination of freshwater, estuarine, and coastal ecosystems. Due to the extreme toxicity, especially the endocrine disruptive characteristic, the use of TBT in small boats had been prohibited in many countries since the mid-1980s. The International Maritime Organization called for a global treaty that bans the application of TBT-based paints starting in 2003, and total prohibition by 2008. However, present and future restrictions will unfortunately not immediately remove TBT and its degradation products from the aquatic environment, since these compounds are reversibly adsorbed to the sediments where they persist (Clark et al., 1988; Unger et al., 1988; Hoch, 2001), particularly under anaerobic conditions with persistence estimated at decades (Dowson et al., 1996; Gadd, 2000). In Spain (Üveges et al., 2007) and Japan (Harino et al., 2007), TBT concentrations in bottom sediments did not decline after regulation or restriction of the use of the compound. Jiang et al. (2001) performed an extensive study of the presence of butyltin compounds in several of China's aquatic environments and reported the highest TBT concentration of 976.9 ng Sn/L. Wide distribution, high hydrophobicity, and persistence of TBT have raised concern about its bioaccumulation in aquatic organisms, especially fish and molluscs (Albalat et al., 2002; Bryan et al., 1993; Fent, 1991; Hu et al., 2014; Kannan et al., 1995; Rantakokko et al., 2006; Yamada et al., 1993). Fish and molluscs can directly take up TBT from the surroundings, e.g., water and sediments, by skin or ventilator organs like gills. A bioconcentration factor (BCF) of 107 (wet weight) for TBT in minnow (*Phoxinus*

Abbreviations: BCF, bioconcentration factor; CI, coloration index; EDCs, endocrine-disrupting chemicals; E_2, 17β-estradiol; GSI, gonadosomatic index; GPI, gonopodial index; PPARs, peroxisome proliferator-activated receptors; PBS, phosphate buffered saline; RXRs, retinoid X receptors; T, testosterone; TBT, tributyltin; TBTCl, tributyltin chloride.

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http://dx.doi.org/10.1016/j.aquatox.2015.03.015
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phoxinus) embryos has been reported (Fent, 1991). The average intake of organotin compounds from foodstuffs was estimated in a Finnish market basket, and the results showed that the fish basket contained the largest number of different organotin compounds (Rantakokko et al., 2006).

The best documented example of endocrine disruption of TBT in vivo is the imposex of female gastropods, characterized by the development of additional male sex organs (penis and/or vas deferens and prostate tissue) on females (Blaber, 1970; Smith, 1981; Horiguchi et al., 1997). Several studies have shown that TBT acts as an inhibitor of cytochrome P450 aromatase (down-regulates the aromatase gene expression and/or inhibits the aromatase activity) (Bettin et al., 1996; Cooke, 2002; Lyssimachou et al., 2006; Saitoh et al., 2001). Aromatase is responsible for the conversion of androgen testosterone (T) into estrogen 17β-estradiol (E_2) (i.e., aromatization) (Simpson et al., 1994). It had been proposed that the TBT-induced imposex of female gastropods was mediated by increased androgen levels of whole animals due to reduced aromatase gene expression/activity (Bettin et al., 1996; Oehlmann et al., 1996; Schulte-Oehlmann et al., 1995; Spooner et al., 1991). Because sex steroids also play a main role in the regulation of sex differentiation and sexual characteristics of teleosts, TBT pollution is also suspected of having adverse effects on fish species. However, in contrast to the extensive literature dealing with the negative impacts of TBT in gastropods (reviewed by Horiguchi, 2006; Matthiessen and Gibbs, 1998; Titley-O'Neal et al., 2011), information on possible effects of TBT on reproductive system of fish is still relatively scarce, particularly in vivo and at environmentally relevant levels (Grzyb et al., 2003; Haubruge et al., 2000; McAllister and Kime, 2003; Mochida et al., 2007; Rurangwa et al., 2002; Santos et al., 2006; Shimasaki et al., 2003). On the other hand, studies on endocrine disruption of TBT, particularly with regard to steroidogenesis, have focussed mainly on gonadal reproductive steroids (Saitoh et al., 2001), since the gonads are considered to be the major source of steroid hormones in the body. However, local steroidogenesis at other sites, especially in the brain, is also very important (MacLusky and Naftolin, 1981). The accumulation of TBT in the central nervous system indicates the potential for toxic effects of TBT on neuroendocrine regulatory centers (Bryan et al., 1993; Horiguchi et al., 2002; Li et al., 2010; Zhang et al., 2008). Until now, limited data exist on possible effects and consequences of TBT causing aromatase suppression in nervous system (Lyssimachou et al., 2006).

Guppies are ovoviviparous fish with a short reproductive period and seasonally independent sperm count, body coloration, gonad size, sexual behavior, and reproductive rate (Houde, 1997). Gonad differentiation and the development of secondary sexual characteristics occur mainly during the juvenile period, and the fish reach full sexual maturity in approximately 3 months. The brightly colored adult male guppies perform distinct courtship behavior, and the anal fin develops into a copulatory organ (the gonopodium) for internal fertilization. Our previous study demonstrated that the male guppies are suitable for studying the effects of endocrine disrupting chemicals (EDCs) on the reproductive system due to the strong visibility and biological relevance of its secondary sex characteristics (gonopodium and pigmentation) (Tian et al., 2012).

In the present study, we conducted a systematic investigation on the reproductive endocrine toxicology of tributyltin at environmentally relevant concentrations in male guppies (*Poecilia reticulata*). First, the response of systemic levels of E_2 and T to TBT exposure in the male guppies was quantified to determine if the typical endocrine-disrupting effects of TBT on gastropods that were seen in previous studies also exist in male fish. Second, a series of biomarkers, including testis size (gonadosomatic index, GSI), length of the gonopodium (gonopodial index, GPI), gonopodium structure, and body coloration (coloration index, CI) was selected to determine the adverse effects of TBT exposure on sexual characteristics, due to the imbalance of sex steroids. Finally, the effects of TBT on mRNA levels of two gene isoforms of aromatase in the brain and frequencies of posturing, sigmoid display, and chase activities were analysed to determine if the changes in mRNA expression of aromatase at the molecular level were associated with a disturbance of reproductive behavior of the individuals.

2. Material and methods

2.1. Fish exposure and sample protocol

Four-month-old adult Red Albino guppies (*P. reticulata*) (2.3 ± 0.2 cm standard length and 0.25 ± 0.05 g wet weight for males; 3.2 ± 0.2 cm standard length and 0.80 ± 0.22 g wet weight for females) were obtained from a dealer in Shanghai, China.

Following acclimation to the laboratory for 2 weeks, guppies were exposed to tributyltin chloride (TBTCl, 96% purity, Lancaster, Lancaster, CA, USA) at nominal concentrations of 5, 50, and 500 ng/L. A control group (dechlorinated tap water) completed the exposure design. Each group of fish (25 females and 30 males) was kept in 50 L aquaria containing 30 L dechlorinated tap water and the appropriate TBT dose using a semi-static toxicity test (10 L water was renewed daily to maintain a constant TBT concentration). The water temperature was maintained at 26 ± 1 °C, and the pH was 7.3 ± 0.2. The photoperiod was 14 h light and 10 h dark. During the experiment, the guppies were fed twice daily with freshly hatched Artemia nauplii and commercial fish feed. Feces and remaining food were removed daily.

Effects of TBT on sexual behavior of the males were assessed after 5, 10, 15, 20, and 25 days of exposure. After 28 days of exposure, 26 male guppies randomly selected from each group were anesthetized in MS-222 (Sigma–Aldrich, St. Louis, MO, USA), and efforts were made to minimize suffering. The body length, body weight, and length of the gonopodium were recorded for the subsequent determination of the GPI ($n = 26$), and the structure of the gonopodium was observed. To determine the GSI, the weight of the testis ($n = 16$) was measured and related to the body weight. The head ($n = 6$) was sampled, immediately frozen in liquid nitrogen, and stored at −80 °C for the quantification of aromatase mRNA by real-time PCR. After the fish ($n = 8$) were imaged for the subsequent determination of the CI, the skin was sampled to determine the carotenoid content ($n = 8$), and then the whole fish (skin and tail fin excluded, $n = 6$) was immediately frozen in liquid nitrogen and stored at −80 °C until the sex steroid levels were measured. All experiments were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Ocean University of China.

2.2. Detection of the sex steroid levels by radioimmunoassay

The whole fish (skin and tail fin excluded) was homogenized in 5 μL of phosphate buffered saline (PBS) per 1 mg of fish tissue. The homogenate was extracted with diethyl ether and centrifuged at $5920 \times g$ for 8 min at 4 °C. And then the supernatant was collected, incubated at 50 °C to remove the diethyl ether, and re-dissolved in PBS (containing 1% bovine serum albumin) for the measurement of the sex steroids.

The T and E_2 levels were detected by radioimmunoassay using commercial kits from the Beijing North Institute of Biological Technology, China. The hormone levels were measured according to the manufacturer's instructions. The assay detection limits were 0.01 ng/mL for T and 0.50 pg/mL for E_2. The inter- and intra-assay coefficients of variation were <10% and <15%, respectively. The

Table 1
Nucleotide sequences of primers used for real-time PCR and product sizes.

Gene	GenBank accession no.	Primer sequence (5′#4′3#)	Amplicon size (bp)
cyp19a	JQ768368	GAGAGAAATGGAGCAGGCAGATAAG CCAGCACGCATTGCCTCAC	110
cyp19b	AY395692	CACACTCTTCATCAGCCTCTTCTTC GCTGTATCATCCAAAACGGTGC	105
β–*actin*	EU143772	GCCTATCTACGAGGGCTACGC TTGATGTCACGCACGATTTCC	150
elf–α	EF408829	CGAGATTGGCGGCATTGG GTTAGGGGGGGCGAAAGTG	91

cross-reactivity of the T antibody to dihydrotestosterone, estriol, E_2, androstenedione, and progesterone was 1.1×10^{-2}%, 2×10^{-15}%, 2.1×10^{-2}%, 1.2×10^{-5}%, and 3.2×10^{-2}%, respectively. The cross-reactivity of the E_2 antibody to estriol, progesterone, and T was 1.6×10^{-2}%, 1.0×10^{-2}%, and 1.0×10^{-2}%, respectively.

2.3. Analysis of the secondary sexual characteristics

To determine the influence of TBT exposure on the secondary sexual characteristics of male guppies, the GPI, gonopodium structure, and CI were analyzed using the method described by Toft and Baatrup (2001). The gonopodium was swung forward, and the length of the gonopodium was measured and related to the body length to determine the GPI. The gonopodium was observed under a dissecting microscope and imaged by a Nikon Coolpix S640 digital camera (Nikon Corporation, Tokyo, Japan) to analyze the structure changes, and a digital image of the left side of the fish was also captured to analyze the coloration of the body. Both the orange-colored area and the whole body area (fins excluded) measured by digital image analysis using Image Tool Version 3.0 and used to quantify the CI, which was determined by calculating the orange-colored area as a percentage of the whole body area (fins excluded).

The carotenoid content in the skin was tested according to the method described by Britton (1985), with minor modifications. The skin (head and fins excluded) was removed from the body with surgical instruments and allowed to dry for several minutes before it was weighed. And then the skin was stored at −80 °C until the carotenoids were extracted with 2 mL of acetone for 3 times. After centrifugation at 1480 × *g* for 2 min at 4 °C, the supernatant was collected and analyzed. The absorbance spectrum of the pigments was obtained from a spectrophotometric scan of the pigment extract using a UV-2800 spectrometer (Unico Shanghai Instruments Co., Ltd., Shanghai, China), with acetone used as a blank control. The carotenoid content was determined from the absorbance of the extract at the peak of absorption using the extinction coefficient $E^{1\%}_{1cm} = 2350$. The carotenoid content per mg of skin was calculated as the total skin carotenoid content of each male divided by the skin weight.

2.4. Quantification of sexual behavior

One day before sexual behavior assay, 6 un-transparent oblong net chests (length × width × height = 15 cm × 10 cm × 10 cm) were temporarily placed in each aquarium, and each male guppy was transferred to one chest. After 12 h, a female was placed in one chest to be paired with the male fish, and male sexual behavior toward the female was recorded for 10 min using a Nikon Coolpix S640 digital camera (Nikon Corporation, Tokyo, Japan) to quantify the frequency of each behavior. Sexual behavior was classified as "posturing" (male moves into female's field of vision and stays to attract her attention), "sigmoid display" (male's body arches to an "S" or "C" form and vibrates while he swims sideways to display the sexually attractive nuptial colors), and "chase" (male follows female and comes into position for a copulation) (Farr, 1976). One person analyzed all data to minimize artificial effects.

2.5. Quantification of aromatase mRNA by real-time PCR

Isolation of total RNA from the brain was carried out using the phenolic reagent TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Extracted RNA was measured by spectrometry at $OD_{260/280}$ prior to treatment with gDNA Eraser (Takara Bio Inc., Shiga, Japan). Equal amounts of RNA (1 μg) were reverse-transcribed into cDNA in 20 μL reactions containing 1 × PrimeScript® Buffer 2 (for Real Time), 1.0 μL PrimeScript® RT Enzyme Mix I, and 1.0 μL RT Primer Mix (Takara Bio Inc., Shiga, Japan). Reverse transcriptions were conducted in a PCR Thermal cycler Dice TP600 (Takara Bio Inc., Shiga, Japan) at 37 °C for 15 min and terminated for 5 s at 85 °C.

Primers were designed for the specific amplification of *cyp19a*, *cyp19b*, β-*actin*, and *elf*-α according to the sequences published in GenBank (Table 1). BLAST and multiple alignment programs of DNAMAN were used to analyze the guppy *cyp19a* and *cyp19b* cDNA sequence. The percentage of identity of the two gene isoforms of guppy *aromatase* is 68.32%, and primers were designed to non–conserved sequence regions based on the alignments. β-*Actin* and *elf*-α transcripts were used together as housekeeping genes to standardize the results and eliminate variations in mRNA and cDNA quantity and quality. Parallel PCR reactions were conducted to amplify the target and reference gene cDNA. Real-time PCR was performed using the Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen, Carlsbad, CA, USA) and an Eppendorf Mastercycler ep *RealPlex*^{4} (Eppendorf, Wesseling-Berzdorf, Germany) in a final volume of 20 μL. The final PCR reactions contained: 1 × Platinum® SYBR® Green qPCR SuperMix-UDG (*Taq* polymerase included), 0.4 μM of each primer, 0.4 μL ROX reference dye, and 4.0 μL first-strand cDNA (template). The thermal profile was 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Melting curve analysis (95 °C for 15 s, 60 °C for 60 s, followed by a slow ramp to 95 °C for 15 s) was applied to all reactions to ensure homogeneity of the reaction products. Neither β-*actin* nor *elf*-α levels were affected by any of the experimental conditions in the study. The relative target gene expression levels were normalized to the geometric mean of the β-*actin* and *elf*-α expression levels following the formula $2^{-\Delta\Delta Ct}$ and plotted on a logarithmic scale.

2.6. Statistics

All data are presented as the mean ± standard deviation. Prior to the parametric analysis, the assumptions of normality and the homogeneity of the variances were examined using probability plots and normality tests. All of the data met the assumptions of normality. Multiple comparisons were assessed using a one-way ANOVA followed by a Tukey post-hoc test (equal variances) or a Games-Howell post-hoc test (unequal variances). For sexual behavior analysis, a two-way ANOVA was performed first, since

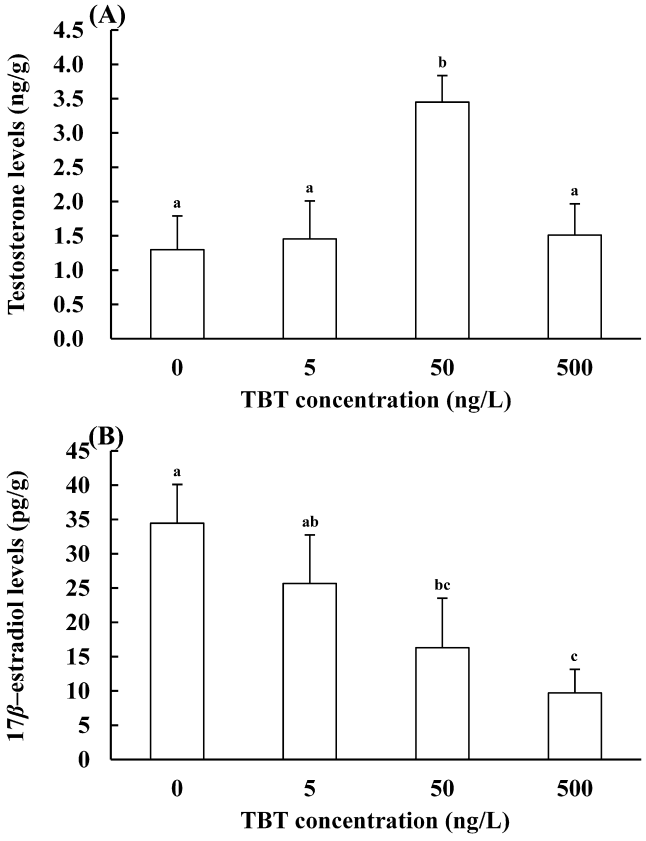

Fig. 1. Sex steroid levels in male guppies exposed to 5, 50, and 500 ng/L TBT for 28 days. (A) T levels. (B) E_2 levels. The data are presented as the mean ± standard deviation ($n = 6$), and values not sharing a common letter denote statistically significant differences according to a one-way ANOVA ($P < 0.05$).

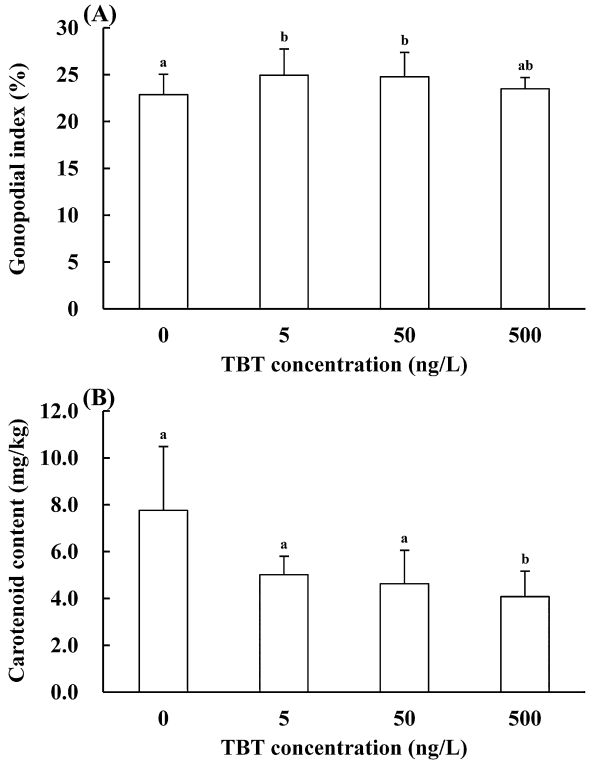

Fig. 2. GPI and carotenoid content in male guppies exposed to 5, 50, and 500 ng/L TBT for 28 days. (A) GPI. (B) Carotenoid content per mg of total skin. The data are presented as the mean ± standard deviation ($n = 26$ for GPI and $n = 8$ for carotenoid content, respectively), and values not sharing a common letter denote statistically significant differences according to a one-way ANOVA ($P < 0.05$).

concentration and duration effects of TBT treatment were investigated. Pearson's correlation coefficient was used to calculate the relationship between GPI values (from the six individuals of which sex hormone levels were quantified) and T levels. A *t*-test analysis was conducted to evaluate the difference between *cyp19a* and *cyp19b* mRNA of the control group. Throughout the experiment, the effects and differences were declared to be significant when their associated *P* values were less than 0.05 ($P < 0.05$).

3. Results

3.1. Effects of TBT on systemic levels of sex steroids

Treatment with 50 ng/L TBT caused a highly significant increase in systemic levels of T of male guppies, while T levels were not changed by 5 ng/L or 500 ng/L TBT treatment (Fig. 1A). There appears to be a concentration-related decrease in systemic levels of E_2 after TBT treatment (Fig. 1B).

3.2. Effects of TBT on GSI

In the control group, the testis made up less than 5% of the body weight, and the GSI of the male fish was unaffected by TBT treatment (data not shown).

3.3. Effects of TBT on GPI and gonopodium structure

The GPI of the control fish was 22.88 ± 2.07%, with an average gonopodium length of 0.525 ± 0.061 cm, and the males exposed to 5 ng/L and 50 ng/L TBT had increased GPIs compared to the controls (Fig. 2A), whereas the structure of the gonopodium was unaffected by TBT treatment (data not shown). Significant positive correlation of GPI values with T levels was found ($r = 0.474$, $P = 0.019$).

3.4. Effects of TBT on CI and carotenoid content

CI analysis revealed that an average of 49.87 ± 7.83% of the body surface was covered with orange spots in the control group and that CI was unaffected by TBT exposure (data not shown).

The skin pigment extracts exhibited two distinct maximum absorption peaks at 446 nm and 468 nm, the former being the tallest. Accordingly, the carotenoid content was determined from the absorbance of the extract at 446 nm. The mean carotenoid content in the skin was decreased by 500 ng/L TBT exposure (Fig. 2B).

3.5. Effects of TBT on reproductive behavior

A two-way ANOVA was conducted that examined the effects of exposure concentration and duration on reproductive behavior of male guppies. There was a significant interaction between the effects of TBT concentration and duration on posturing, but not on sigmoid display or chase (Table 2).

Significant effects of TBT concentration were found by two-way ANOVA for all these three kinds of reproductive behaviour. Especially for chase, inhibiting effects were observed in treatments with 5 ng/L, 50 ng/L, and 500 ng/L TBT compared to the control (Table 2). Significant inhibitions on sigmoid display and posturing were found in animals exposed to TBT of the two highest and the highest concentration, respectively (Table 2).

Significant effects of exposure duration were found for posturing and sigmoid display frequencies but not for chase activities. Compared to the animals exposed for 5 days, male guppies treated

Table 2
The two-way analysis of variance (*P* value).

	Posturing	Sigmoid display	Chase
Concentration	0.001 500 ng/L < 0 ng/L	<0.0005 500 ng/L = 50 ng/L <0 ng/L	<0.0005 500 ng/L = 50 ng/L = 5 ng/L <0 ng/L
Duration	0.011 25 d < 5 d	<0.0005 25 d = 15 d < 5 d	0.125
Concentration × duration	0.026	0.320	0.156

with TBT for 25 days exerted decreased posturing activities (Table 2). Male guppies exposed to TBT for 25 days and 15 days showed similar frequencies of sigmoid display but lower sigmoid display activities than individuals treated for 5 days (Table 2), and these inhibiting effects could be seen in multiple comparisons of differences between the 5 ng/L TBT-treated groups for different durations by one-way ANOVA (Fig. 3).

3.6. Effects of TBT on aromatase gene expression in the brain

Baseline variability of *cyp19a* and *cyp19b* gene expression was first compared in the control group. In the brain of male guppies, *cyp19b* mRNA levels were significantly higher than *cyp19a* mRNA levels (Fig. 4).

Both *cyp19a* and *cyp19b* gene expression in the brain of male guppies was downregulated after a 28 day exposure to TBT (Fig. 4).

4. Discussion

The possibility that reproductive system of fish regulated by sex hormones might be affected by TBT interfering with the production of sex hormones as an aromatase inhibitor should be considered. In this study, the adverse effects and intrinsic toxicological properties of TBT in male fish were evaluated using aromatase expression in the brain at the molecular level, sex steroid contents at the biochemical level, primary and secondary sexual characteristics at the tissue/organ level, and reproductive behavior at the individual level as apical endpoints.

The results in the present study indicated that after the 28-day exposure to 50 ng/L TBT, the systemic levels of T were increased in the adult male guppies, and elevated E_2 levels were observed concomitantly. T plays important roles in the development and maintenance of sexual characteristics in male guppies (Bayley et al., 2002; Borg, 1994). Elevated GPIs were observed in the 5 ng/L and 50 ng/L TBT treated groups, while the structure of the gonopodium was unaffected by TBT exposure. The development of a gonopodium, which is critical for sperm transfer, is a male-specific secondary sexual characteristic. Both the juvenile males and females of the family Poeciliidae have an anal fin that is essentially the same in structure. During the growth and development of the female, both the anal fin and body increase in size proportionately, and growth continues after sexual maturity has been reached. In contrast, in the male, the anal fin and the spinal column undergo a series of hormone-dependent changes that result in the development of gonopodium, which is used as copulatory organ. At full maturity, the growth of both the body and gonopodium ceases. Consistent with the results of this study, Pandey (1969) found that female guppies exposed to exogenous androgen develop gonopodia, and Bayley et al. (2002) demonstrated that significantly smaller gonopodia develop in males treated with antiandrogens. Considering the important role of androgen T in gonopodium development, as well as the significant correlation between GPI values and T levels, we proposed that the increase in length of the gonopodium (at least in the 50 ng/L TBT exposure group) was caused by the rise of T levels.

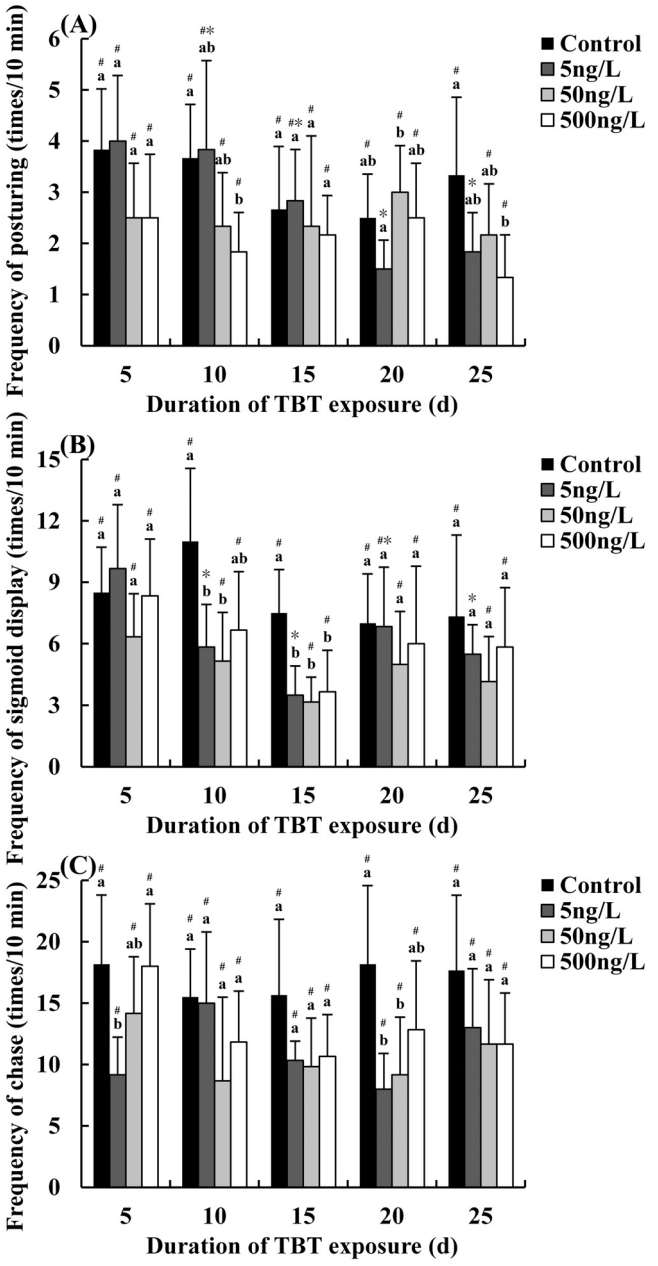

Fig. 3. Frequencies of reproductive behavior in male guppies exposed to 5, 50, and 500 ng/L TBT for 5, 10, 15, 20, and 25 days. (A) Posturing. (B) Sigmoid display. (C) Chase. The data are presented as the mean ± standard deviation ($n = 6$). Values not sharing a common letter denote statistically significant differences between treatments with a given exposure duration according to a one-way ANOVA ($P < 0.05$). Values not sharing a common symbol denote statistically significant differences between treatments with a given TBT concentration according to a one-way ANOVA ($P < 0.05$).

It is worth to notice that there were non-linear responses of T levels and GPI to increasing TBT levels, with no upregulation of these two endpoints observed in the highest concentration group. This may be related to the complicated endocrine disrupting effects of TBT proposed recently. TBT is well known to show masculinizing effects as a competitive inhibitor of aromatase (Bettin et al., 1996; Cooke, 2002; Lyssimachou et al., 2006; Saitoh et al., 2001). However, there were studies indicating that other biological actions, including increase in the number of retinoid X receptors (RXRs) and peroxisome proliferator-activated recep-

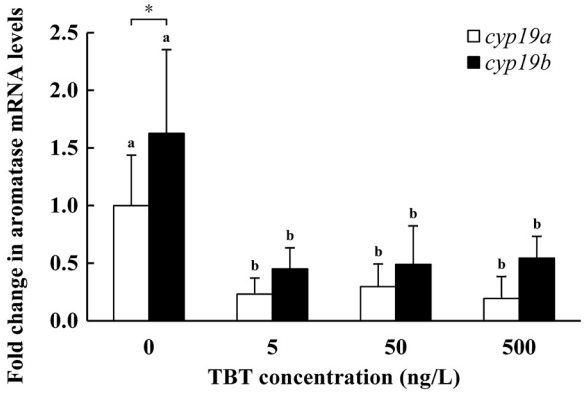

Fig. 4. Relative mRNA levels of aromatase in the brain of male guppies exposed to 5, 50, and 500 ng/L TBT for 28 days. Fold change (Y axis) represents the expression of *cyp19a*/*cyp19b* mRNA relative to that of the control group (expression of *cyp19a* is set to 1). The data are presented as the mean ± standard deviation ($n = 6$). Values of a given gene isoform not sharing a common letter denote statistically significant differences according to a one-way ANOVA ($P < 0.05$). The asterisk indicates statistically significant difference between *cyp19a* and *cyp19b* of the control group according to a *t*-test analysis (*$P < 0.05$).

tors (PPARs) (Carfi' et al., 2008Grün et al., 2006; Kanayama et al., 2005; Nakanishi, 2008; Nakanishi et al., 2005; Nishikawa et al., 2004; Urushitani et al., 2011), abnormal lipid accumulation and decreased T esterification (Gooding et al., 2003; Grün et al., 2006; Inadera and Shimomura, 2005; Janer et al., 2006, 2007; Kanayama et al., 2005; Sternberg and LeBlanc, 2006; Zhang et al., 2013), and suppressed testicular T biosynthesis (Heidrich et al., 2001; McVey and Cooke, 2003; Ohno et al., 2005) might be involved in the reproductive toxicity mechanisms of TBT. For example, in mammals, it is considered that TBT acts as an unspecific, but significant inhibitor of T biosynthesis, since activities of steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (Heidrich et al., 2001; McVey and Cooke, 2003), 17β-hydroxysteroid dehydrogenase (McVey and Cooke, 2003; Ohno et al., 2005), and cytochrome P450 17 alpha-hydroxylase (Ohno et al., 2005) were decreased by TBT exposure. It is possible that, in the 500 ng/L TBT group, the accumulation of T by the inhibition of aromatase is counteracted by the inhibition effects of T biosynthesis, thereby resulting in an unaffected T levels and GPI values.

Guppies show a complex color pattern polymorphism, and several strains have been established accordingly. Guppies also show remarkable sexual dichroism. Male guppies display a sexual coloration that is absent in females. Red Albino guppies were chosen as the experimental organisms in this study. They are the albino form of a red strain, and the male guppies of this strain have sexually attractive orange coloring. Fish can quickly change color by dispersing or aggregating the pigment in the chromatophores (physiological color change) or more slowly by altering the amount of pigment and/or the number of chromatophores (morphological color change) (Stepien, 1986). A pronounced fading of the display coloration was caused by blocking the synthesis of sex steroids in adult male guppies by hypophysectomy (Pandey, 1969a). Display coloration was partially restored by the subsequent treatment of these hypophysectomized males with exogenous androgen (Pandey, 1969b). The sexual colorations of guppies were altered by exposure to E_2, 4-*tert*-octylphenol, vinclozolin, *p,p′*-DDE, and flutamide (Baatrup and Junge, 2001; Bayley et al., 2002; Toft and Baatrup, 2001, 2003). Therefore, the bright coloration of the male-specific secondary sexual characteristics in guppies is considered a sensitive visual indicator for the analysis of the effects of EDCs on the male reproductive phenotypes. Since maintenance of bright coloration in male guppies is androgen–dependent, it is reasonable to assume that the increase in T levels of TBT-treated individuals observed in this study may potentially elevate CI of male guppies. However, no alteration was found in the CI values between the treatment groups and the control. Surprisingly, we found that compared the control, the mean carotenoid content in the total skin of the 500 ng/L TBT treated fish was reduced.

Carotenoids have been identified as the main pigments involved in the expression of yellow, orange, and red colors in fish (Hudon, 1994). During sexual development, carotenoids are continually deposited in the skin of the male fish to produce the sexually attractive nuptial colors. In addition to their function in the production of display colors, carotenoid pigments also serve a variety of physiological roles, not only as precursors for vitamin A but also as antioxidants, free-radical scavengers, immune-system enhancers, and cancer inhibitors (McGraw and Ardia, 2003; Vershinin, 1999). Thus, allocation trade-offs between the immune system and sexual traits could be brought about by competition for carotenoids that can be deposited in ornaments or used as antioxidants in support of immune functions (Alonso-Alvarez et al., 2004; Lozano, 1994; Peters et al., 2004). It needs to be confirmed that if the decrease of carotenoid content in the skin is caused by a significant cost of immune investment under TBT stress. On the other hand, although it is often assumed that red, orange, and yellow colors in animals are derived from carotenoid pigments, there are other pigments that confer similar colors on animals. It was reported that red pteridine pigments (drosopterins) were displayed in the skins of male guppies in addition to carotenoids (Grether et al., 2001). The relationship(s) between synthesis of sexual hormones, expression of sexual signal pigments (pteridine production and carotenoid availability), and maintenance of conspicuous colors still need to be further researched.

Another important feature of T is its control of activating and maintaining reproductive behavior in males (Borg, 1994). Reproductive behavior of castrated male fish was recovered by treatment of androgens, which could also induce reproductive behavior in females (Borg, 1994). The reproductive biology and behavior of guppies has been extensively described (Baerends et al., 1955; Houde, 1997). Male courtship consists of an elaborate display in which the male orientates himself in front of the female, a feature known as posturing, and contorts to an S-shape or C-shape to show female his sexually attractive colors, a feature known as sigmoid display, and then the male chases the female to achieve copulation (Farr, 1976). Basically, frequencies of posturing, sigmoid display, and chase activities were either unaffected or inhibited by exposure to TBT at different concentrations for different durations. The inhibiting effects on sigmoid display and chase were observed even in the 50 ng/L treatment groups, of which T levels were significantly increased. In mammalian and avian vertebrate groups, androgens act as controlling agents on male courtship behavior by their conversion to estrogens by cytochrome P450 aromatase in well-defined brain regions (Balthazart et al., 1996, 2003, 2004; Lephart, 1996). Two structurally and functionally distinct P450 aromatase isoforms, which are products of separate gene loci (*cyp19a* and *cyp19b*), have been identified in most teleosts (Chang et al., 2005; Sawyer et al., 2006; Strobl-Mazzulla et al., 2005). Our results revealed that *cyp19b* gene was expressed at a significantly higher level in the brain of male guppies than *cyp19a*. In Nile tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*), and pejerrey fish (*Odontesthes bonariensis*), it has been demonstrated that CYP19B is the predominant neural isoform and is expressed at high levels compared to CYP19A, which is mainly present in the gonad (Chang et al., 2005; Sawyer et al., 2006; Strobl-Mazzulla et al., 2005). Although the exact regulatory effects of aromatase in the brain of male teleosts on reproductive behavior is still largely unknown, several lines of evidence suggest that it probably plays roles in organismal reproductive performance and health similar to those of mammals and birds (Forlano et al., 2001; Goto-Kazeto et al.,

2004; Hallgren et al., 2006; Schlinger et al., 1999). For example, Hallgren et al. (2006) demonstrated that sexual behaviors in male guppies were inhibited by partial inhibition of aromatase activity in the brain with the use of a competitive aromatase inhibitor fadrozole, indicating a connection between activity of aromatase in the brain and male reproductive behavior in guppies. In this study, both the effects of TBT concentration and of TBT exposure duration on reproductive behavior of male guppies were assessed, since quantification of sexual behavior can be conducted without harming the fish, while aromatase mRNA levels in response to TBT exposure at different concentrations was determined only after 28 days of exposure. Considering the significant effects of TBT concentration on both activities of three kinds of reproductive behavior and gene expression levels of two gene isoforms encoding aromatase in brain, we suggest that TBT treatment inhibited gene expression of *cyp19a* and *cyp19b* in brain of male guppies and thus caused the disturbance of male reproductive behavior even at environmentally relevant concentrations (5–500 ng/L).

It has been reported that TBT altered the sex ratio toward males in zebrafish (*D. rerio*) (McAllister and Kime, 2003; Santos et al., 2006) and Japanese flounder (*Paralichthys olivaceus*) (Shimasaki et al., 2003), suppressed spermatogenesis in mummichog (*Fundulus heteroclitus*) (Mochida et al., 2007), African catfish (*Clarias gariepinus*) (Rurangwa et al., 2002), common carp (*Cyprinus carpio*) (Rurangwa et al., 2002), herring (*Clupea harengus*) (Grzyb et al., 2003), cuvier (*Sebastiscus marmoratus*) (Zhang et al., 2009), and guppies (*P. reticulata*) (Haubruge et al., 2000), and decreased GSI in female cuvier (*Sebastiscus marmoratus*) (Zhang et al., 2007). The masculinizing effects of TBT on sex differentiation in fish correlate well with the induction of imposex in aquatic invertebrates exposed to environmental TBT. However, sex reversal could be induced only after chronic exposure to TBT or exposure at particular periods in development when individuals may be especially sensitive to hormone titre (McAllister and Kime, 2003; Santos et al., 2006; Shimasaki et al., 2003). In this study, change of reproductive behavior was earlier than that of GSI in response to TBT. Specifically, multiple comparisons of treatments with different TBT concentrations for a given exposure duration indicated that significant inhibition of chase frequency was already observed when males were exposed to TBT at the lowest concentration of 5 ng/L for the shortest duration of 5 days, while GSI was unaffected by 28 days of 5, 50, and 500 ng/L TBT treatment. There was one article addressing adverse effects of TBT in fish sexual behavior (Nakayama et al., 2004), and our present study further elucidated how TBT acted on the neural system and suppressed sexual behavior. Predatory behavior in *S. marmoratus* and antipredator behavior in threespine stickleback (*Gasterosteus aculeatus* L.) were influenced by TBT exposure, and these deteriorations in behavior are believed to be caused by depressed effects on the nervous system (Wibe et al., 2001; Yu et al., 2013). Behavior is an ecologically relevant tool for detecting pollution in the environment since it reflects the consequence of physiological, biochemical, and/or molecular alterations caused by contamination (Peakall, 1996). In addition, behavior prevents unnecessary destruction and suffering of animals. Courtship rate of males is a reliable indicator of fertility/reproductive success. In general, females are attracted to males with high courtship display (Farr, 1980; Nicoletto, 1993), and significant correlations were detected between sperm number and male sexual behavior (Matthews et al., 1997). Thus, we suggest reproductive behavior of males to be an ecologically relevant integrative biomarker sensitively and visually indicating endocrine disruption of short-term TBT exposure in fish.

In summary, TBT induced a shift of the androgen–estrogen balance in favor of androgen in adult male guppies and thus resulted in elevated GPI. To the best of our knowledge, this is the first report describing effects of TBT on secondary sexual characteristics of teleosts. The inhibiting effects of TBT on sexual behavior (potential biomarker in male fish) were observed, which is suggested to be induced by decrease of aromatase gene expression in the brain.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31202001) and the Natural Science Foundation of Shandong Province (ZR2012CQ010).

References

Albalat, A., Potrykus, J., Pempkowiak, J., Porte, C., 2002. Assessment of organotin pollution along the Polish coast (Baltic Sea) by using mussels and fish as sentinel organisms. Chemosphere 47, 165–171.
Alonso-Alvarez, C., Bertrand, S., Devevey, G., Gaillard, M., Prost, J., Faivre, B., Sorci, G., 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am. Nat. 164, 651–659.
Baatrup, E., Junge, M., 2001. Antiandrogenic pesticides disrupt sexual characteristics in the adult male guppy *Poecilia reticulata*. Environ. Health Perspect 109, 1063–1070.
Baerends, G.P., Brouwer, R., Waterbolk, H.T., 1955. Ethological studies on *Lebistes reticulatus* (Peters) I. An analysis of the male courtship pattern. Behaviour 8, 249–334.
Balthazart, J., Baillien, M., Charlier, T.D., Cornil, C.A., Ball, G.F., 2003. The neuroendocrinology of reproductive behavior in Japanese quail. Domest. Anim. Endocrinol. 25, 69–82.
Balthazart, J., Baillien, M., Cornil, C.A., Ball, G.F., 2004. Preoptic aromatase modulates male sexual behavior: slow and fast mechanisms of action. Physiol. Behav. 83, 247–270.
Balthazart, J., Foidart, A., Absil, P., Harada, N., 1996. Effects of testosterone and its metabolites on aromatase-immunoreactive cells in the quail brain: relationship with the activation of male reproductive behavior. J. Steroid Biochem. Mol. Biol. 56, 185–200.
Bayley, M., Junge, M., Baatrup, E., 2002. Exposure of juvenile guppies to three antiandrogens causes demasculinization and a reduced sperm count in adult males. Aquat. Toxicol. 56, 227–239.
Bettin, C., Oehlmann, J., Stroben, E., 1996. TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. Helgolander Meeresu. 50, 299–317.
Blaber, S.J.M., 1970. The occurrence of a penis-like outgrowth behind the right tentacle in spent females of *Nucella lapillus* (L.). Proc. Malac. Soc. Lond. 39, 231–233.
Borg, B., 1994. Androgens in teleost fishes. Comp. Biochem. Physiol. C Pharmacol. Toxicol. 109, 219–245.
Britton, G., 1985. General carotenoid methods. Methods Enzymol. 111, 113–149.
Bryan, G.W., Bright, D.A., Hummerstone, L.G., Burt, G.R., 1993. Uptake, tissue distribution and metabolism of ^{14}C-labelled tributyltin (TBT) in the dog-whelk, *Nucella lapillus*. J. Mar. Biol. Assoc. U.K. 73, 889–912.
Carfì', M., Croera, C., Ferrario, D., Campi, V., Bowe, G., Pieters, R., Gribaldo, L., 2008. TBTC induces adipocyte differentiation in human bone marrow long term culture. Toxicology 249, 11–18.
Chang, X., Kobayashi, T., Senthilkumaran, B., Kobayashi-Kajura, H., Sudhakumari, C.C., Nagahama, Y., 2005. Two types of aromatase with different encoding genes: tissue distribution and developmental expression in nile tilapia (*Oreochromis niloticus*). Gen. Comp. Endocrinol. 141, 101–115.
Clark, E.A., Sterritt, R.M., Lester, J.N., 1988. The fate of tributyltin in the aquatic environment. Environ. Sci. Technol. 22, 600–604.
Cooke, G.M., 2002. Effect of organotins on human aromatase activity in vitro. Toxicol. Lett. 126, 121–130.
Dowson, P.H., Bubb, J.M., Lester, J.N., 1996. Persistence and degradation pathways of tributyltin in freshwater and estuarine sediments. Estuarine Coastal Shelf Sci. 42, 551–562.
Farr, J.A., 1976. Social facilitation of male sexual behavior, intrasexual competition, and sexual selection in the guppy, *Poecilia reticulata* (Pisces: Poeciliidae). Evolution 30, 707–717.
Farr, J.A., 1980. Social behavior patterns as determinants of reproductive success in the guppy, *Poecilia reticulata* Peters (Pisces: Poeciliidae): an experimental study of the effects of intermale competition, female choice, and sexual selection. Behaviour 74, 38–91.
Fent, K., 1991. Bioconcentration and elimination of tributyltin chloride by embryos and larvae of minnows *Phoxinus phoxinus*. Aquat. Toxicol. 20, 147–158.
Forlano, P.M., Deitcher, D.L., Myers, D.A., Bass, A.H., 2001. Anatomical distribution and cellular basis for high levels of aromatase activity in the brain of teleost fish: aromatase enzyme and mRNA expression identify glia as source. J. Neurosci. 21, 8943–8955.

Gadd, G.M., 2000. Microbial interactions with tributyltin compounds: detoxification accumulation and environmental fate. Sci. Total Environ. 258, 119–127.
Gooding, M.P., Wilson, V.S., Folmar, L.C., Marcovich, D.T., LeBlanc, G.A., 2003. The biocide tributyltin reduces the accumulation of testosterone as fatty acid esters in the mud snail (*Ilyanassa obsoleta*). Environ. Health Perspect. 111, 426–430.
Goto-Kazeto, R., Kight, K.E., Zohar, Y., Place, A.R., Trant, J.M., 2004. Localization and expression of aromatase mRNA in adult zebrafish. Gen. Comp. Endocrinol. 139, 72–84.
Grether, G.F., Hudon, J., Endler, J.A., 2001. Carotenoid scarcity: synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulata*). Proc. Biol. Sci. 268, 1245–1253.
Grün, F., Watanabe, H., Zamanian, Z., Maeda, L., Arima, K., Chubacha, R., Gardiner, D.M., Iguchi, T., Kanno, J., Blumberg, B., 2006. Endocrine disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. Mol. Endocrinol. 20, 2141–2155.
Grzyb, K., Rychlowski, M., Biegniewska, A., Skorkowski, E.F., 2003. Quantitative determination of creatine kinase release from herring (*Clupea harengus*) spermatozoa induced by tributyltin. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 134, 207–213.
Hallgren, S.L.E., Linderoth, M., Olsén, K.H., 2006. Inhibition of cytochrome P450 brain aromatase reduces two male specific sexual behaviours in the male Endler guppy (*Poecilia reticulata*). Gen. Comp. Endocrinol 147, 323–328.
Harino, H., Yamamoto, Y., Eguchi, S., Kawai, S., Kurokawa, Y., Arai, T., Ohji, M., Okamura, H., Miyazaki, N., 2007. Concentrations of antifouling biocides in sediment and mussel samples collected from Otsuchi Bay Japan. Arch. Environ. Contam. Toxicol. 52, 179–188.
Haubruge, E., Petit, F., Gage, M.J., 2000. Reduced sperm count in guppies (*Poecilia reticulata*) following exposure to low levels of tributylin and bisphenol A. Proc. Roy. Soc. Lond. 267, 2333–2337.
Heidrich, D.D., Steckelbroeck, S., Klingmuller, D., 2001. Inhibition of human cytochrome P450 aromatase activity by butyltins. Steroids 66, 763–769.
Hoch, M., 2001. Organotin compounds in the environment–an overview. Appl. Geochem. 16, 719–743.
Horiguchi, T., 2006. Masculinization of female gastropod mollusks induced by organotin compounds: focusing on mechanism of actions of tributyltin and triphenyltin for development of imposex. Environ. Sci. 13, 77–87.
Horiguchi, T., Kojima, M., Kaya, M., Matsuo, T., Shiraishi, H., Morita, M., Adachi, Y., 2002. Tributyltin and triphenyltin induce spermatogenesis in ovary of female abalone *Haliotis gigantea*. Mar. Environ. Res. 54, 679–684.
Horiguchi, T., Shiraishi, H., Shimizu, M., Morit, M., 1997. Imposex in sea snails: caused by organotin (tributyltin and triphenyltin) pollution in Japan: a survey. Appl. Organomet. Chem. 11, 451–455.
Houde, A.E., 1997. Sex Color, and Mate Choice in Guppies. Princeton University Press, Princeton.
Hu, Y., Gong, X., Xu, Y., Song, X., Liu, H., Deng, X., Ru, S., 2014. Risk assessment of butyltins based on a fugacity-based food web bioaccumulation model in the Jincheng Bay mariculture area: I Model development. Environ. Sci. Process. Impacts 16, 1994–2001.
Hudon, J., 1994. Biotechnological applications of research on animal pigmentation. Biotechnol. Adv. 12, 49–69.
Inadera, H., Shimomura, A., 2005. Environmental chemical tributyltin augments adipocyte differentiation. Toxicol. Lett. 159, 226–234.
Janer, G., Lyssimachou, A., Bachmann, J., Oehlmann, J., Schulte-Oehlmann, U., Porte, C., 2006. Sexual dimorphism in esterified steroid levels in the gastropod *Marisa cornuarietis*: the effect of xenoandrogenic compounds. Steroids 71, 435–444.
Janer, G., Navarro, J.C., Porte, C., 2007. Exposure to TBT increases accumulation of lipids and alters fatty acid homeostasis in the ramshorn snail *Marisa cornuarietis*. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 146, 368–374.
Jiang, G.B., Zhou, Q.F., Liu, J.Y., Wu, D.J., 2001. Occurrence of butyltin compounds in the waters of selected lakes: rivers and coastal environments from China. Environ. Pollut. 115, 81–87.
Kanayama, T., Kobayashi, N., Mamiya, S., Nakanishi, T., Nishikawa, J., 2005. Organotin compounds promote adipocyte differentiation as agonists of the peroxisome proliferator-activated receptor c/retinoid X receptor pathway. Mol. Pharmacol. 67, 766–774.
Lephart, E.D., 1996. A review of brain aromatase cytochrome P450. Brain Res. Brain Res. Rev. 22, 1–26.
Kannan, K., Tanabe, S., Iwata, H., Tatsukawa, R., 1995. Butyltins in muscle and liver of fish collected from certain Asian and Oceanian countries. Environ. Pollut. 90, 279–290.
Li, B., Wang, C., Yu, A., Chen, Y., Zuo, Z., 2010. Identification of differentially expressed genes in the brain of *Sebastiscus marmoratus* in response to tributyltin exposure. Aquat. Toxicol. 99, 248–255.
Lozano, G.A., 1994. Carotenoids, parasites, and sexual selection. Oikos 70, 309–311.
Lyssimachou, A., Jenssen, B.M., Arukwe, A., 2006. Brain cytochrome P450 aromatase gene isoforms and activity levels in atlantic salmon after waterborne exposure to nominal environmental concentrations of the pharmaceutical ethynylestradiol and antifoulant tributyltin. Toxicol. Sci. 91, 82–92.
Matthews, I.M., Evans, J.P., Magurran, A.E., 1997. Male display rate reveals ejaculate characteristics in the Trinidadian guppy *Poecilia reticulata*. Proc. Biol. Sci. 264, 695–700.
MacLusky, N.J., Naftolin, F., 1981. Sexual differentiation of the central nervous system. Science 211, 1294–1302.
Matthiessen, P., Gibbs, P.E., 1998. Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in molluscs. Environ. Toxicol. Chem. 17, 37–43.
McAllister, B.G., Kime, D.E., 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). Aquat. Toxicol. 65, 309–316.
McGraw, K.J., Ardia, D.R., 2003. Carotenoids immunocompetence, and the information content of sexual colors: an experimental test. Am. Nat. 162, 704–712.
McVey, M.J., Cooke, G.M., 2003. Inhibition of rat testis microsomal 3beta-hydroxysteroid dehydrogenase activity by tributyltin. J. Steroid Biochem. Mol. Biol. 86, 99–105.
Mochida, K., Ito, K., Kono, K., Onduka, T., Kakuno, A., Fujii, K., 2007. Molecular and histological evaluation of tributyltin toxicity on spermatogenesis in a marine fish: the mummichog (*Fundulus heteroclitus*). Aquat. Toxicol. 83, 73–83.
Nakanishi, T., Nishikawa, J., Hiromori, Y., Yokoyama, H., Koyanagi, M., Takasuga, S., Ishizaki, J., Watanabe, M., Isa, S., Utoguchi, N., 2005. Trialkyltin compounds bind retinoid X receptor to alter human placental endocrine functions. Mol. Endocrinol. 19, 2502–2516.
Nakanishi, T., 2008. Endocrine disruption induced by organotin compounds; organotins function as a powerful agonist for nuclear receptors rather than an aromatase inhibitor. J. Toxicol. Sci. 33, 269–276.
Nakayama, K., Oshima, Y., Yamaguchi, T., Tsuruda, Y., Kang, I.J., Kobayashi, M., Imada, N., Honjo, T., 2004. Fertilization success and sexual behavior in male medaka, *Oryzias latipes*, exposed to tributyltin. Chemosphere 55, 1331–1337.
Nicoletto, P.F., 1993. Female sexual response to condition-dependent ornaments in the guppy *Poecilia reticulata*. Anim. Behav. 46, 441–450.
Nishikawa, J.I., Mamiya, S., Kanayama, T., Nishikawa, T., Shiraishi, F., Horiguchi, T., 2004. Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. Environ. Sci. Technol. 38, 6271–6276.
Oehlmann, J., Stroben, E., Schulte-Oehlmann, U., Bauer, B., Fioroni, P., Markert, B., 1996. Tributyltin biomonitoring using prosobranchs as sentinel organisms. Fresenius J. Anal. Chem. 354, 540–545.
Ohno, S., Nakajima, Y., Nakajin, S., 2005. Triphenyltin and tributyltin inhibit pig testicular 17beta-hydroxysteroid dehydrogenase activity and suppress testicular testosterone biosynthesis. Steroids 70, 645–651.
Pandey, S., 1969a. Effects of hypophysectomy on the testis and secondary sex characters of the adult guppy (*Poecilia reticulata* Peters). Can. J. Zool. 47, 775–781.
Pandey, S., 1969b. Effects of methyl testosterone on the testis and secondary sex characters of the hypophysectomized adult guppy *Poecilia reticulata* Peters. Can. J. Zool. 47, 783–786.
Pandey, S., 1969. The role of pituitary and gonadal hormones in the differentiation of testis and secondary sex characters of the juvenile guppy *Poecilia reticulata* Peters. Biol. Reprod. 1, 272–281.
Peakall, D.B., 1996. Disrupted patterns of behavior in natural populations as an index of ecotoxicity. Environ. Health Perspect. 104, 331–335.
Peters, A., Delhey, K., Denk, A.G., Kempenaers, B., 2004. Trade-offs between immune investment and sexual signaling in male mallards. Am. Nat. 164, 51–59.
Rantakokko, P., Kuningas, T., Saastamoinen, K., Vartiainen, T., 2006. Dietary intake of organotin compounds in Finland: a market-basket study. Food Addit. Contam. 23, 749–756.
Rurangwa, E., Biegniewska, A., Slominska, E., Skorkowski, E.F., Ollevier, F., 2002. Effect of tributyltin on adenylate content and enzyme activities of teleost sperm: a biochemical approach to study the mechanisms of toxicant reduced spermatozoa motility. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 131, 335–344.
Saitoh, M., Yanase, T., Morinaga, H., Tanabe, M., Mu, Y.M., Nishi, Y., Nomura, M., Okabe, T., Goto, K., Takayanagi, R., Nawata, H., 2001. Tributyltin or triphenyltin inhibits aromatase activity in the human granulosa-like tumor cell line KGN. Biochem. Biophys. Res. Commun. 289, 198–204.
Santos, M.M., Micael, J., Carvalho, A.P., Morabito, R., Booy, P., Massanisso, P., Lamoree, M., Reis-Henriques, M.A., 2006. Estrogens counteract the masculinizing effect of tributyltin in zebrafish. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 142, 151–155.
Sawyer, S.J., Gerstner, K.A., Callard, G.V., 2006. Real-time PCR analysis of cytochrome P450 aromatase expression in zebrafish: gene specific tissue distribution sex differences, developmental programming, and estrogen regulation. Gen. Comp. Endocrinol. 147, 108–117.
Schlinger, B.A., Greco, C., Bass, A.H., 1999. Aromatase activity in the hindbrain vocal control region of a teleost fish: divergence among males with alternative reproductive tactics. Proc. R. Soc. Lond. B 266, 131–136.
Schulte-Oehlmann, U., Bettin, C., Fioroni, P., Oehlmann, J., Stroben, E., 1995. Marisa cornuarietis (Gastropoda, Prosobranchia): a potential TBT bioindicator for freshwater environments. Ecotoxicology 4, 372–384.
Shimasaki, Y., Kitano, T., Oshima, Y., Inoue, S., Imada, N., Honjo, T., 2003. Tributyltin causes masculinization in fish. Environ. Toxicol. Chem. 22, 141–144.
Simpson, E.R., Mahendroo, M.S., Means, G.D., Kilgore, M.W., Hinshelwood, M.M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C.R., Michael, M.D., Mendelsen, C.R., Bulun, S.E., 1994. Aromatase cytochrome P450: the enzyme responsible for estrogen biosynthesis. Endocr. Rev. 15, 342–355.
Smith, B.S., 1981. Tributyltin compounds induce male characteristics on female mud snails *Nassarius obsoletus* = *Ilyanassa obsoleta*. J. Appl. Toxicol. 1, 141–144.

Spooner, N., Gibbs, P.E., Bryan, G.W., Goad, L.J., 1991. The effect of tributyltin upon steroid titres in the female dogwhelk, *Nucella lapillus*, and the development of imposex. Mar. Environ. Res. 32, 37–49.

Stepien, C.A., 1986. Regulation of color morphic patterns in the giant kelpfish, *Heterostichus rostratus* Girard: genetic versus environmental factors. J. Exp. Mar. Biol. Ecol. 100, 181–208.

Sternberg, R.M., LeBlanc, G.A., 2006. Kinetic characterization of the inhibition of acyl coenzyme A: steroid acyltransferases by tributyltin in the eastern mud snail (*Ilyanassa obsoleta*). Aquat. Toxicol. 78, 233–242.

Strobl-Mazzulla, P.H., Moncaut, N.P., López, G.C., Miranda, L.A., Canario, A.V., Somoza, G.M., 2005. Brain aromatase from pejerrey fish (*Odontesthes bonariensis*): cDNA cloning tissue expression, and immunohistochemical localization. Gen. Comp. Endocrinol. 143, 21–32.

Tian, H., Li, Y., Wang, W., Wu, P., Ru, S., 2012. Exposure to monocrotophos pesticide during sexual development causes the feminization/demasculinization of the reproductive traits and a reduction in the reproductive success of male guppies (*Poecilia reticulata*). Toxicol. Appl. Pharmacol. 263, 163–170.

Titley-O'Neal, C.P., Munkittrick, K.R., Macdonald, B.A., 2011. The effects of organotin on female gastropods. J. Environ. Monit. 13, 2360–2388.

Toft, G., Baatrup, E., 2001. Sexual characteristics are altered by 4-*tert*-octylphenol and 17 β-estradiol in the adult male guppy (*Poecilia reticulata*). Ecotoxicol. Environ. Saf. 48, 76–84.

Toft, G., Baatrup, E., 2003. Altered sexual characteristics in guppies (*Poecilia reticulata*) exposed to 17β-estradiol and 4-*tert*-octylphenol during sexual development. Ecotoxicol. Environ. Saf. 56, 228–237.

Unger, M.A., MacIntyre, W.G., Huggett, R.J., 1988. Sorption behaviour of tributyltin on estuarine and freshwater sediments. Environ. Toxicol. Chem. 7, 907–915.

Urushitani, H., Katsu, Y., Ohta, Y., Shiraishi, H., Iguchi, T., Horiguchi, T., 2011. Cloning and characterization of retinoid X receptor (RXR) isoforms in the rock shell *Thais clavigera*. Aquat. Toxicol. 103, 101–111.

Üveges, M., Rodríguez-González, P., García Alonso, J.I., Sanz-Medel, A., Fodor, P., 2007. Isotope dilution analysis mass spectrometry for the routine measurement of butyltin compounds in marine environmental and biological samples. Microchem. J. 85, 115–121.

Vershinin, A., 1999. Biological functions of carotenoids-diversity and evolution. Biofactors 10, 99–104.

Wibe, A.E., Nordtug, T., Jenssen, B.M., 2001. Effects of bis(tributyltin) oxide on antipredator behavior in threespine stickleback *Gasterosteus aculeatus* L. Chemosphere 44, 475–481.

Yamada, H., Tateishi, M., Takayanagi, K., 1993. Bioaccumulation of organotin compounds in the red sea bream (*Pagrus major*) by two uptake pathways: dietary uptake and uptake from water. Environ. Toxicol. Chem. 13, 1415–1422.

Yu, A., Wang, X., Zuo, Z., Cai, J., Wang, C., 2013. Tributyltin exposure influences predatory behavior, neurotransmitter content and receptor expression in *Sebastiscus marmoratus*. Aquat. Toxicol. 128–129, 158–162.

Zhang, J., Zuo, Z., Wang, n-name>ChenC., 2008. Tributyltin exposure causes brain damage in *Sebastiscus marmoratus*. Chemosphere 73, 337–343.

Zhang, J., Zuo, Z., He, C., Wu, D., Chen, Y., Wang, C., 2009. Inhibition of thyroidal status related to depression of testicular development in *Sebastiscus marmoratus* exposed to tributyltin. Aquat. Toxicol. 94, 62–67.

Zhang, J., Zuo, Z., Chen, Y., Zhao, Y., Hu, S., Wang, C., 2007. Effect of tributyltin on the development of ovary in female cuvier (*Sebastiscus marmoratus*). Aquat. Toxicol. 83, 174–179.

Zhang, J., Zuo, Z., Xiong, J., Sun, P., Chen, Y., Wang, C., 2013. Tributyltin exposure causes lipotoxicity responses in the ovaries of rockfish, *Sebastiscus marmoratus*. Chemosphere 90, 1294–1299.