

RESEARCH REPORT

Exposure to tributyltin chloride induces penis and vas deferens development and increases RXR expression in females of the purple snail (*Plicopurpura pansa*)**D Domínguez-Ojeda¹, AE Rojas-García², ML Robledo-Marenco², BS Barrón-Vivanco², IM Medina-Díaz²**¹*Escuela Nacional de Ingeniería Pesquera, Universidad Autónoma de Nayarit, Bahía de Matanchén Km. 12, San Blas, 63740 Nayarit, México*²*Laboratorio de Contaminación y Toxicología Ambiental, Universidad Autónoma de Nayarit, Av. de la Cultura s/n, Col. Los Fresnos, 63155 Tepic, Nayarit, México*

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Abstract

Tributyltin (TBT) and its derivatives are widely used as antifouling paints for ships, resulting in their being released into the marine environment. Aquatic invertebrates, particularly marine gastropods, are extremely sensitive to TBT and undergo changes in the imposition of male secondary sex characteristics in response to exposure. This study aimed to evaluate the development of imposex and the expression of the retinoid X receptor (RXR) in tissues of *Plicopurpura pansa* (males and females) exposed to tributyltin chloride (TBTCI). The histological results showed a penis-like structure in imposex female and an undeveloped vas deferens that lacked circular muscular layers. TBTCI treatment increased the messenger RNA (mRNA) of RXR in females with imposex. The highest level of mRNA RXR was found in the digestive gland and penis-forming area in females under *in vivo* exposure compared with control females. These results indicate that TBTCI modulates mRNA levels of RXR in females. mRNA RXR in imposex females and females exposed to TBTCI only was similar to that of males, indicating that RXR might contribute to the development of imposex. To our knowledge, this study is the first to show that TBTCI induces imposex and biphallia in this snail species, and that this effect is accompanied by an increase in RXR expression.

Key Words: tributyltin; imposex; retinoid X receptor (RXR); purple snail**Introduction**

Organotin compounds have been widely used as antifouling paints for ships and fishing nets since the 1960s, resulting in their continuous release into the marine environment (De Mora, 1996; Fent, 1996; Champ, 2000). Tributyltin (TBT) and its derivatives are endocrine disruptors that induce deregulation of vertebrate and invertebrate endocrine systems (Golub and Doherty, 2004). Aquatic invertebrates, particularly marine gastropods, are extremely sensitive to organotin compounds such as TBT. Consequently, they undergo changes in imposition of male secondary sex characteristics in response to exposure (Maguire, 2000; Oetken *et al.*, 2004; Sternberg *et al.*, 2010), which is caused by a decrease in aromatase

activity (the enzyme that converts androgens to estrogens) (Matthiessen and Gibbs, 1998; McAllister and Kime, 2003). This worldwide phenomenon represents one of the most detrimental consequences of pollution by anthropogenic chemicals and has led these compounds to being banned from antifouling paints in a number of countries; however, organotin compounds remain in the environment (Nishikawa, 2006).

To date, very low concentrations of TBT have been shown to induce imposex in marine gastropods (Smith, 1980, 1981; Bryan *et al.*, 1993; Horiguchi *et al.*, 1997; Matthiessen and Gibbs, 1998; Nishikawa, 2006; Chacón *et al.*, 2007; Oehlmann *et al.*, 2007). These abnormalities are the result of a masculinization process by which male sex organs develop a notable penis and vas deferens. In certain species, the growth of the vas deferens disrupts the structure and function of the oviducts, preventing normal breeding activity and causing population decline (Bryan *et al.*, 1986; Gibbs and Bryan, 1986; Nakanishi, 2008). At present, the effects associated with TBT have been reported in 268 species of

Corresponding author:
Irma Martha Medina Díaz
Laboratorio de Contaminación y Toxicología Ambiental
Secretaría de Investigación y Posgrado
Universidad Autónoma de Nayarit
63155 Tepic, Nayarit, México
E-mail: irmartha@hotmail.com

Table 1 Body size and weight of female and male purple snails used in flow-through exposure experiments (October to March, 2011)

	Control females	Control males	TBTCl-exposed females	TBTCl-exposed males
Shell height (cm)	3.14±0.346	3.16±0.249	3.085±0.33	3.185±0.27
Shell weight (g)	4.87±1.61	5.6±1.19	4.85±1.8	6.22±1.40

Mean ± Standard deviation (SD). There was no statistical differences between size and weight with exposure to tributyltin chloride (TBTCl).

intertidal gastropods worldwide, belonging to the orders Vetigastropoda, Mesogastropoda, and Neogastropoda. Neogastropoda is the most affected order, which contains 214 species, of which 101 are part of the Muricidae family (Titley-O'Neal *et al.*, 2011). The purple snail (*Plicopurpura pansa*) is part of this affected group of species. This snail is an intertidal carnivorous gastropod that inhabits rocky intertidal beaches exposed to strong wave action. It is distributed in the Pacific Ocean, from the northwestern Mexican coast to northern Peru (Keen, 1971). In addition, this gastropod possesses both economic and cultural importance.

Alternative mechanisms by which TBT induces imposex in gastropods include the following: 1) increase in androgen levels as a result of the TBT-mediated inhibition of aromatase (Spooner *et al.*, 1991; Bettin *et al.*, 1996); 2) TBT-mediated inhibition of the excretion of androgen sulfate conjugates, with a consequent increase in androgen levels (Ronis

and Mason, 1996); 3) an increase in the level of an alanine-proline-glycine-tryptophan amide neuropeptide in response to TBT, and 4) TBT interference in the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia (Féral and Le Gall, 1983).

Another important mechanism proposed to induce imposex development is through the retinoid X receptor (RXR). This receptor is a member of the nuclear receptor superfamily of ligand-activated transcription factors that have been characterized in a wide variety of metazoan phyla and is highly conserved (Bouton *et al.*, 2005). RXR play a central role in a variety of nuclear signaling pathway, have multiple physiological functions, and play key roles in embryo patterning and organogenesis in mammals. RXR is known to act both as a ligand-dependent transcription factor and as a common hetero- or homodimer partner for many non-steroid

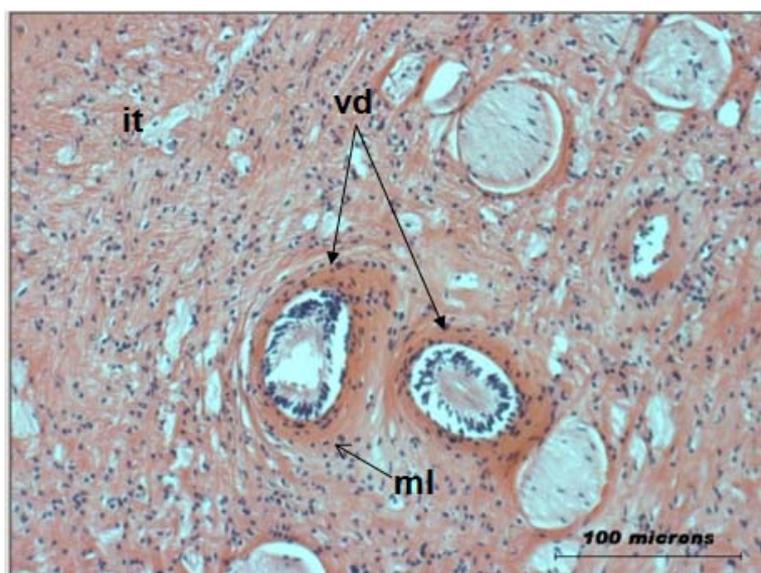


Fig. 1 Hematoxylin and eosin semi-thin cross-section of the male penis of the purple snail 2 months after treatment with 10 µg Sn/L of Tributyltin chloride (TBTCl). Abbreviations: it, interstitial tissue; ml, muscle layer; vd, vas deferens.

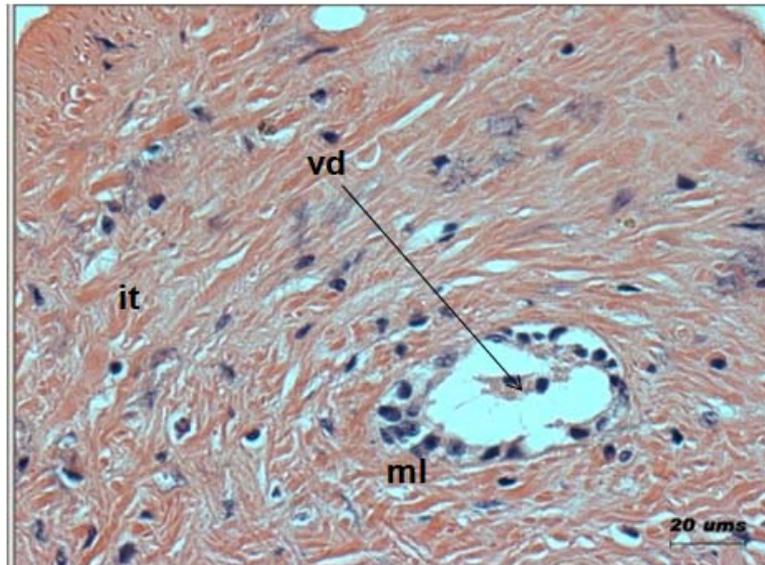


Fig. 2 Hematoxylin and eosin cross-section of the penis-like structure (2 mm in length) that developed behind the right tentacle of a female purple snail 2 months after treatment with 10 µg Sn/L of Tributyltin chloride (TBTCI). Abbreviations: it, interstitial tissue; ml, muscle layer; vd, vas deferens.

nuclear receptors. Because 9-cis retinoic acid effectively induces imposex, RXR may play an important role in the development of imposex in gastropods (Mangelsdorf and Evans 1995; Morris-Kay 1997; Bouton *et al.*, 2005; Stenberg *et al.*, 2008). Recently, Horiguchi *et al.* (2010) suggested that RXR might be involved in organotin-mediated induction of male-type genitalia (penis and vas deferens) in female rock shells (*Thais clavigera*).

Although imposex studies have been completed in several gastropods, the development of imposex by TBT in the purple snail (*Plicopurpura pansa*) has not been previously described. The aim of the present study was to evaluate the development of imposex and expression in the *RXR* gene (mRNA) of female and male *P. pansa* exposed to Tributyltin chloride (TBTCI).

Materials and Methods

Collection of specimens

Experiments were performed with a gonochoristic prosobranch species, the purple snail, *Plicopurpura pansa* Neogastropoda: Thaididae. In March 2010, live female and male purple snails were collected by hand at low tide at Olas Altas, Mazatlán, Sinaloa, Mexico (23°12' 29.87" N, 106°25' 43.67" W). The specimens were immediately transported to the laboratory in plastic cage containing seawater from the collection site. Once at the laboratory, the specimens were reared for 2 weeks in a laboratory aquarium to acclimate to artificial seawater (Red Sea Salt®, Israel). The purple snails were fed with pieces of squid (*Loligo* spp.).

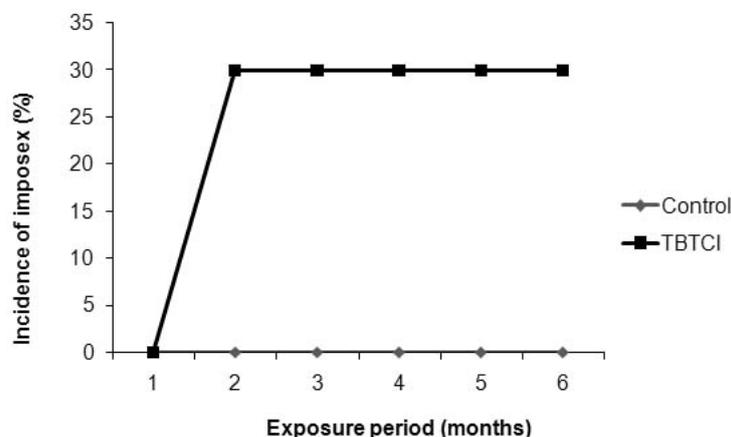


Fig. 3 Temporal change in the incidence of imposex in female purple snails exposed to 10 µg Sn/L of Tributyltin (TBTCI) for 6 months in a flow-through system.

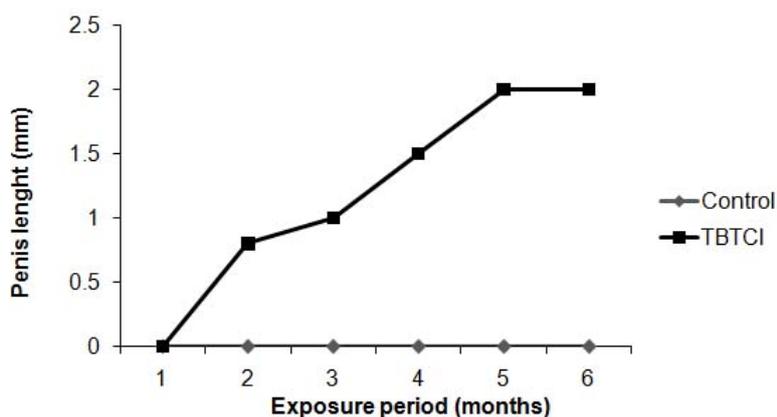


Fig. 4 Temporal change in the average penis length of female purple snails exposed to 10 µg Sn/L of Tributyltin (TBTCl) for 6 months in a flow-through system.

Histological procedure

The soft tissues (penis and penis-like structure) of males and imposex females were extracted from the shell and were fixed with 10% formaldehyde for 12 h. After dehydration with ethanol (70, 80, 90, 96, and 100%), the tissues were immersed in xylene and then embedded in paraffin and sliced into 7 µm sections. The paraffin-embedded sections were then stained with Hematoxylin and eosin and examined under a light microscope.

In vivo experiments with TBT

Before the experiments, the purple snails were manipulated to allow the selection of females, males, and imposex females. Males were identified by the penis, which is located behind the right cephalic tentacle, a common feature among neogastropods. This organ has a characteristic inverted-cedilla form with a 2 mm width that becomes thicker at the base; females were identified by the absence of this characteristic and the presence of organs such as albumen and capsule glands (Domínguez-Ojeda *et al.*, 2009). Shell length was measured with a vernier caliper with 0.05 mm precision and weight was determined on an Adam digital scale ($d = 0.01$ g). The purple snails (50 total, including 20 females, 10 imposex-exhibiting females, and 20 males). First, the snails were evenly divided into two experimental groups by sex, with two groups containing 10 females and two groups containing 10 males. One group of each sex was treated with Tributyltin chloride (TBTCl) (10 µg Sn/L, obtained from LC₅₀), and the remaining group was used as the control. The group of imposex-exhibiting females was employed to compare what happens in the wild. Stock solutions of TBTCl (Sigma-Aldrich Co., St. Louis, MO, USA, 96 %) for the exposure experiments were diluted with ethanol. Females with imposex ($n = 10$) were treated under the same conditions as the control groups. Each experimental group of purple snails was maintained for 6 months in an 8 L glass bottle (control females, exposed females, imposex-exhibiting females,

control males, and exposed males) with a flow-through system of artificial seawater (Read Sea Salt®, Israel) by means of an aeration pump. The temperature of the experimental seawater was maintained at 25 ± 1 °C and a salinity of 38 ± 2 Salt per unit (SPU). The purple snails in the two exposure groups were removed each month for imposex examination (presence or absence of penis) and for assays of RXR gene expression in several tissues. These snails were dissected to remove the ovary or testis, digestive gland, penis-forming area or penis, and head ganglia according to sex (male or female). Then, each sample, containing the respective tissue of 10 individuals according to sex, was prepared. The survival rate of the purple snails in all of the experimental groups was 100 %. Table 1 shows the body sizes of the purple snails utilized in the experiment.

Isolation of total RNA

Total RNA was prepared from purple snail tissue using Trizol reagent, according to manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA was quantified spectrophotometrically at OD₂₆₀, and purity was assessed by measuring the OD₂₆₀/OD₂₈₀ ratio. RNA integrity was evaluated by electrophoresing RNA samples in 1 % agarose gel. Complementary DNA (cDNA) was prepared from 4 µg of total RNA, using the SuperScript Pre-amplification System for First Strand Synthesis and oligodT.

Assay for RXR gene expression

To assay RXR gene expression in various tissues, 1 µl of cDNA was subjected to Polymerase chain reaction (PCR). The following program was used: denaturation at 94 °C for 10 min; 35 cycles of PCR with denaturation at 94 °C for 15 sec; annealing at 45.5 °C for 30 sec, and extension at 72 °C for 30 sec. Forward and reverse primers were used: RXR, 5'-GATTCTGGAGCCGAGATTG-3' and 5'-TGGCTCTTCTGGGCATCA-3' (Horiguchi *et al.*, 2010) (product size, 150 pb), respectively. To

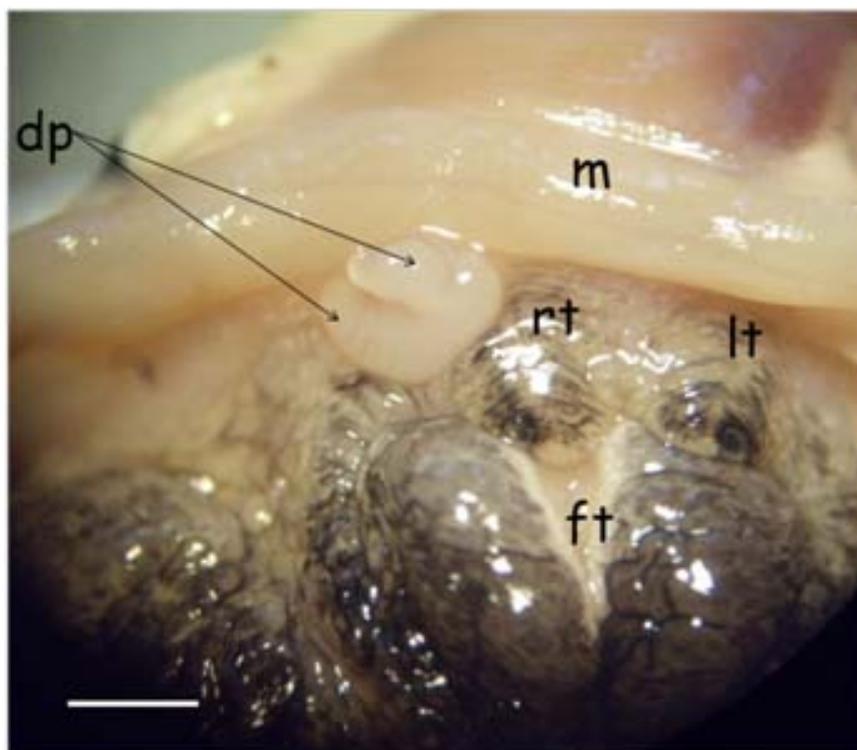


Fig. 5. Imposed female purple snail with biphallia after 6 months exposure to 10 µg Sn/L of Tributyltin (TBTCI). Abbreviations: db, double penis; m, mantle; rt, right tentacle; lt, left tentacle; ft, foot. Scale bar: 2 mm.

normalize *RXR* gene expression in the respective tissues, the expression of 18S ribosomal RNA (*18S rRNA*), a housekeeping gene, was used as a reference to observe the effects of upregulated and downregulated genes and was also assayed in these tissues. *RXR* gene expression in each tissue was normalized by dividing the value for *RXR* gene content by the value for *18S rRNA* content. PCR products were electrophoresed on 2 % agarose gel and stained with ethidium bromide to visualize the PCR amplification products. Relative intensity was determined using the Sigma Gel program (Jandel Scientific Software).

Statistical analysis

The statistical significance of any difference in imposex levels in TBTCI-exposed groups compared with the control groups was tested. The statistical significance of the incidence of imposex was determined by the Fisher exact test, and one-factor Analysis of variance (ANOVA) was used for penis length. For *RXR* gene expression in each tissue, we evaluated the statistical significance of the differences between TBTCI-exposed groups and the control groups by Mann-Whitney *U* test.

Results

Histological features of normal male penis and imposed female penis treated with TBTCI

A semi-thin cross-section of the male penis shows that it possesses two vas deferens, smooth

muscle cells surrounding the epithelium, and interstitial cells (Fig. 1). In comparison, the imposed female had an undeveloped vas deferens that lacked circular muscular layers in the interstitial tissues. In addition, the muscle cells and internal epithelium were not visible, possibly because of being at an early stage of differentiation (Fig. 2).

Effects of TBTCI on imposex development in P. pansa

The experiments confirm that TBTCI induces imposex in *P. pansa* females. The incidence of imposex was significantly higher in exposed females compared with control females ($p < 0.001$) (Fig. 3). Female penis length gradually, but significantly, increased during the first 2 months of exposure ($p < 0.01$) (Fig. 4). Furthermore, at month 6 of TBTCI exposure, one of the imposed females presented a double penis, the length of each penis 2 mm (Fig. 5).

Effect of TBTCI on RXR mRNA expression

TBTCI treatment increased the expression of *RXR* mRNA in females with imposex (Fig. 6a). The highest level of *RXR* mRNA expression was found in the digestive gland (3.2-fold) and penis-forming area (2.5-fold) ($p < 0.02$) in TBTCI-treated females compared with control females. However, *RXR* expression was significantly lower in the gonad and head ganglia of imposed females exposed to TBTCI compared with control females ($p < 0.05$) (Fig.

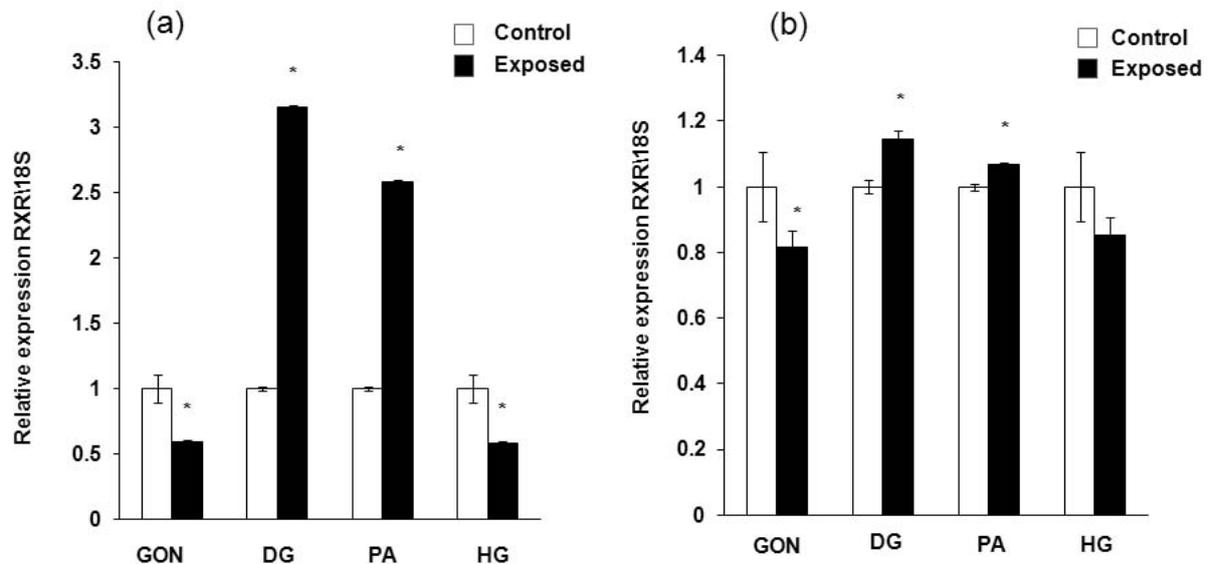


Fig. 6 Expression of RXR mRNA levels in several tissues of (a) *Plicopurpura pansa* imposex females, and (b) females exposed to Tributyltin chloride (TBTCI). Female snails were exposed to TBTCI (10 µg Sn/L) or 0.5 % ethanol (control) during 6 months. Relative expression of RXR mRNA was determined by PCR. Changes in level of expression were determined by normalizing the data to 18S and were expressed relative to the control or to the female without imposex. Data are given as the mean ± Standard deviations (SD) from two experiments performed in triplicate. The asterisk (*) indicates significant differences vs. the control ($p < 0.05$). GON, Gonad; DG, Digestive Gland; PA, Penis Area; HG, Head Ganglia

6a). Less pronounced effects were observed in females without imposex that were treated with TBTCI (Fig. 6b). These results indicate that TBTCI might modulate mRNA levels of *RXR* in females.

In addition, we investigated whether a similar increase in *RXR* mRNA levels was recorded in males treated with TBTCI (Fig. 7). No significant increase in *RXR* gene expression was observed in the digestive gland and penis-forming area of males exposed to TBTCI compared with control males. However, a significant decrease in *RXR* mRNA expression was observed in the gonad, head ganglia, and spermatid gland ($p < 0.05$).

Discussion

In this study, we demonstrated that 10 µg Sn/L TBTCI induces imposex within 2 months in normal female *P. pansa*. This finding indicates that *RXR* might be important in the development of imposex in gastropods. Low concentrations of TBT (<1 ng/L [such as Sn]) have been shown to induce imposex in neogastropods (Gibbs *et al.*, 1988; Gooding *et al.*, 2003). TBT concentrations of this magnitude and greater have been measured in marine environments where imposex occurs (Svavarsson *et al.*, 2001). Thus, there is much evidence implicating TBT as the cause of imposex among prosobranch gastropods. A previous study indicated that induction of imposex and penis growth required 1 and 2 - 3 months, respectively, in *Thais clavigera* (Horiguchi *et al.*, 2010).

This study also demonstrated that 10 µg Sn/L TBTCI causes the penis-like structures observed in the females of treated purple snails to form, with

these structures being histologically recognizable as penises with vas deferens. These structures resembled the organs of morphologically normal males and of imposex females collected from the wild. It is noteworthy that a previous study by our group recorded similar or higher concentrations of TBT in wild *P. pansa* (Domínguez-Ojeda, 2012).

Several examples of intersex and sex reversal have been hypothesized as being induced by environmental chemicals in wildlife. Furthermore, some studies have shown that organotins or ligands for nuclear hormone receptors (retinoid X receptors [RXR]) cause a decrease in the production of Cytochrome P450-dependent aromatase (CYP19) mRNA in human ovarian granulosa cells (Mu *et al.*, 2001). In addition, the RXR homolog in gastropod mollusks has been found to be responsive to 9-*cis* Retinoic acid (RA), with TBT and 9-*cis* RA shown to induce imposex (Nishikawa *et al.*, 2004; Castro *et al.*, 2007). These findings indicate that the transcriptional mechanism for TBT action is conserved across phyla (Iguchi and Katsu, 2008). In this study, *RXR* expression in imposex females and females exposed to TBTCI was similar to that of males. This observation indicates that *RXR* might contribute to imposex development. mRNA levels of *RXR* in TBTCI-exposed females might have been modulated in the population of *P. pansa*, as documented in other studies (Bryan *et al.*, 1986; Gibbs *et al.*, 1991; Huet *et al.*, 1996).

Also, in this study, we observed different values of *RXR* expression in different tissues, in different treatments, and in different sexual categories. These results are according to Horiguchi *et al.* (2010); the authors observed different patterns of

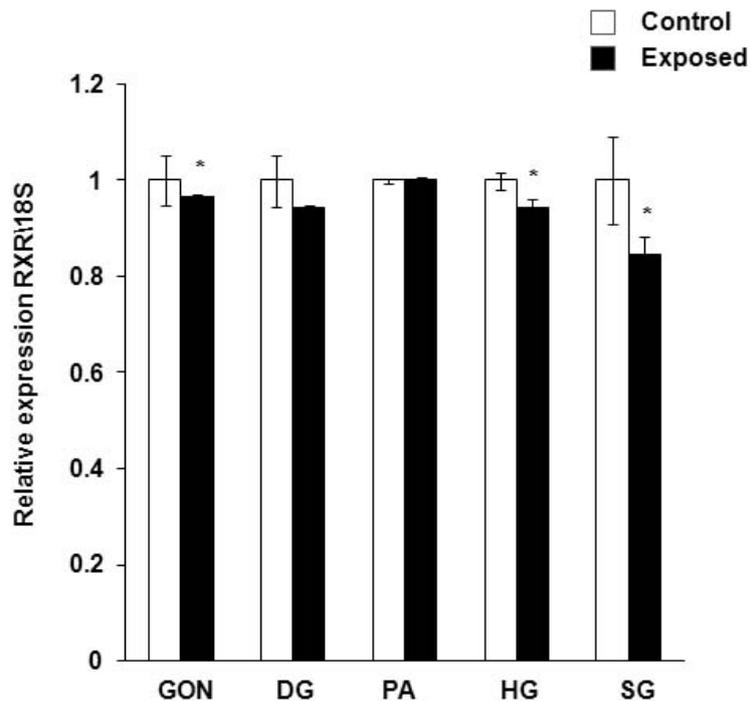


Fig. 7 Effects of Tributyltin chloride (TBTCI) on the RXR mRNA levels in several tissues of *Plicopurpura pansa* males. Male snails were exposed to TBTCI (10 µg Sn/L) or 0.5 % ethanol (control). After 6 months of treatment, relative expression of messenger RNA (RXR mRNA) was determined by PCR. Changes in level of expression were determined by normalizing the data to 18S and were expressed relative to the control. Data are given as the mean ± Standard deviations (SD) from two experiments performed in triplicate. The asterisk (*) indicates significant differences vs. the control ($p < 0.05$). GON, Gonad; DG, Digestive Gland; PA, Penis Area; HG, Head Ganglia; SG, Spermatic Gland.

expression of RXR among tissues analyzed. RXR possesses ubiquitous, or quite widespread, expression patterns, similar to those of other nuclear receptors (RAR β and RAR γ). RXR levels of expression are related with early developmental stages and are tissue-specific. In terms of differential expression, only in some instances does their distribution correlate with specific differentiating cell or tissue types throughout the organism. Although RXR are expressed rather ubiquitously, their expression also depends on epigenetic regulation, e.g., modifications in histones and degree of methylation of CpG dinucleotides (Dollé, 2009; Laursen et al., 2012).

The differences observed in the time required for the development of imposex in different species might be associated with differences in the rate of accumulation of organotins to a level that exceeds the threshold concentration for increased RXR expression in target organs/cells. This parameter might vary according to the characteristics of each species. In addition, some studies have found that TBT increases Testosterone (T) titers in a dose- and time-dependent manner in snails, with administration of T also inducing imposex (Spooner et al., 1991; Stroben et al., 1991).

Although the present study did not demonstrate the mechanisms by which TBTCI gives rise to

imposex in *P. pansa*, it is, to our knowledge, the first to demonstrate that TBTCI induces imposex and biphallia in this species of snail, and that this effect is accompanied by an increase in RXR expression.

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