

## Retinoid X receptor gene expression and protein content in tissues of the rock shell *Thais clavigera*

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Received 3 April 2007; received in revised form 25 June 2007; accepted 26 June 2007

### Abstract

To elucidate the role of retinoid X receptor (RXR) in the development of imposex caused by organotin compounds in gastropod molluscs, we investigated RXR gene expression and RXR protein content in various tissues of male and female wild rock shells (*Thais clavigera*). Quantitative real-time polymerase chain reaction, Western blotting, and immunohistochemistry with a commercial antibody against human RXR  $\alpha$  revealed that RXR gene expression was significantly higher in the penises of males and imposex-exhibiting females than in the penis-forming areas of normal females ( $P < 0.01$  and  $P < 0.05$ , respectively). Western blotting demonstrated that the antibody could detect rock shell RXR and showed that the male penis had the highest content of RXR protein among the analyzed tissues of males and normal females. Immunohistochemical staining revealed nuclear localization of RXR protein in the epithelial and smooth muscle cells of the vas deferens and in the interstitial or connective tissues and epidermis of the penis in males and imposex-exhibiting females. RXR could be involved in the mechanism of induction of male-type genitalia (penis and vas deferens) by organotin compounds in female rock shells.

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**Keywords:** Imposex; Tributyltin; Triphenyltin; Rock shell (*Thais clavigera*); Retinoid X receptor (RXR); Gene expression

### 1. Introduction

Organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) have been used around the world in antifouling paints for ships and fishing nets since the mid-1960s, and their release into marine environments has resulted in worldwide pollution (Goldberg, 1986). Organotin compounds are known to have harmful effects in both vertebrates and invertebrates (e.g., Fent, 1996; Golub and Doherty, 2004). Of the organotin compounds, TBT and TPT, in particular, are very toxic to organisms, including marine species. One of the most typical toxic effects of TBT and TPT on marine organisms is the induction of imposex in meso- and neogastropods. Imposex is defined as an irreversible syndrome imposing male-type genital organs, such as the penis and vas deferens, upon females (Smith, 1971; Bryan et al., 1986). Gastropod imposex is typically induced by very low concentrations of TBT, TPT, or both (e.g., approximately 1 ng/L of TBT;

Bryan et al., 1986, 1987, 1988; Gibbs et al., 1987; Horiguchi et al., 1994, 1997a). Reproductive failure is involved in the severe stages of imposex, because of either oviduct blockage by vas deferens formation or ovarian spermatogenesis, resulting in population decline and/or mass extinction (Gibbs and Bryan, 1986; Gibbs et al., 1988, 1990; Horiguchi et al., 2006). Globally, more than 150 species of gastropod, including the rock shell (*Thais clavigera*), are affected by imposex (Fioroni et al., 1991; Horiguchi et al., 1997b, 2006; Matthiessen et al., 1999). Gastropod imposex is also thought to be a clear manifestation of endocrine disruption in wildlife (Matthiessen and Gibbs, 1998; Matthiessen et al., 1999).

Four mechanisms for the induction of imposex by organotins in gastropods have been proposed: (1) an increase in the levels of androgens such as testosterone as a result of aromatase inhibition by TBT (Bettin et al., 1996); (2) inhibition by TBT of the excretion of sulfate conjugates of androgens (Ronis and Mason, 1996); (3) disturbance by TBT of penis morphogenetic/retrogressive factor released from the pedal/cerebropleural ganglia (Féral and Le Gall, 1983); and (4) an increase in the level of a neuropeptide, APGWamide,

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in response to TBT (Oberdörster and McClellan-Green, 2000). However, one pertinent question is whether gastropods, like vertebrates, have sex steroid hormones. Although estrogen receptor (ER)-like cDNA has been isolated from *Aplysia californica* (Gastropoda: Opisthobranchia), estrogen cannot bind to it, and it is a constitutively activated transcription factor (Thornton et al., 2003). ER-like protein has also been isolated from *T. clavigera*, but estrogen cannot bind to it, either (Kajiwara et al., 2006; Iguchi et al., 2007); like the protein in *A. californica*, it is a constitutively activated transcription factor (Iguchi et al., 2007). A study of fully sequenced invertebrate genomes did not find homologs of the ER and androgen receptor (AR) in invertebrates (Escriva et al., 1997). Thus, it is still unclear whether gastropods have functional steroid hormone receptors such as the AR and ER (Horiguchi, 2006). Additionally, the effects of an aromatase inhibitor (fadrozole; 5 µg/g wet tissue) in combination with testosterone (0.1 µg/g wet tissue) in the induction and/or promotion of the development of imposex appear weak, as indicated by experimental results on the incidences of imposex and penis growth (Iguchi et al., 2007).

On the other hand, Nishikawa et al. (2004) recently found that *T. clavigera* had retinoid X receptor (RXR), similar to humans and other vertebrates and to invertebrates such as ascidians, insects, pulmonates, jellyfish, and sponges (Heyman et al., 1992; Mangelsdorf et al., 1992; Mangelsdorf and Evans, 1995; Kostrouch et al., 1998; Freebern et al., 1999; Kamimura et al., 2000; Devine et al., 2002; Nagatomo et al., 2003; Wiens et al., 2003; Bouton et al., 2005), and that the rock shell RXR binds to both 9-*cis*-retinoic acid (9CRA) and organotins. Nishikawa et al. (2004) demonstrated that a single *in vivo* injection of either 9CRA or TPT into normal female rock shells resulted in the development of imposex a month later, and these researchers put forward a new hypothesis that RXR has an important role in the development of imposex caused by organotins in gastropods.

As the first step toward clarifying the overall modes of action of TBT and TPT, in terms of the involvement of RXR in the development of imposex in gastropods, we investigated RXR gene expression and RXR protein content in various tissues (testis/ovary, digestive gland, ctenidium, penis/penis forming area, and head ganglia) of males, normal females, and imposex-exhibiting females in wild rock shell populations. Localization of RXR protein was also investigated by immunohistochemical staining. If RXR actually has an important role in the development of imposex caused by organotins in gastropods, its gene expression and localization of the protein should be clearly observable in the penises of both males and imposex-exhibiting females in wild rock shell populations.

## 2. Materials and methods

### 2.1. Collection and handling of organisms

For assay for RXR gene expression, both male and female wild rock shells (approximately 300 individuals) were collected at Hiraiso, in Ibaraki Prefecture, Japan (a reference site known to have low levels of contamination with TBT and TPT; less than 10 and 8 ng/g wet wt. for TBT and TPT, respectively;

see Horiguchi et al., 1997a), in December 2003. They were reared in a laboratory aquarium in artificial seawater (Senju Pharmaceutical, Osaka, Japan) for approximately 1 month to remove trace levels of TBT and TPT that may have affected gene expression. The density of rock shells in the aquarium was about 300 individuals/155 L; the water temperature,  $20.3 \pm 0.1$  °C; the pH  $7.94 \pm 0.07$ ; and the salinity,  $34.0 \pm 0.9$  psu; means  $\pm$  standard deviations. These rock shells were fed live mussels (*Septifer virgatus*), which were also collected at Hiraiso. Tissue concentrations of TBT and TPT in the mussels were lower than those in the rock shells (Horiguchi et al., 1997a). Only normal females, and males, were selected and used for the following experiments; that is, the percentage occurrence of imposex among females was approximately 9.5%, a level considered low. The shell heights of the males and normal females used in the experiments were  $22.2 \pm 3.7$  and  $22.7 \pm 2.0$  mm (mean  $\pm$  standard deviation), respectively.

Imposex-exhibiting female rock shells (approximately 50 individuals) were collected at Jogashima, in Kanagawa Prefecture, Japan (a site severely contaminated with TBT and TPT; e.g., tissue concentrations of TBT and TPT in rock shells were 52.8 and 80.2 ng/g wet wt., respectively; see Horiguchi et al., 2000), in March 2004, and the organisms were used immediately for these experiments. The shell height of the imposex-exhibiting females used here was  $26.5 \pm 2.0$  mm (mean  $\pm$  standard deviation). Because of the severe contamination by TBT and TPT at Jogashima, no normal females were collected there; all of the females exhibited imposex (including sterile ones due to oviduct blockage by vas deferens formation).

The rock shells were dissected to remove the testis or ovary, digestive gland, ctenidium, penis or penis-forming area, and head ganglia from the soft tissues according to sex (male, normal female, or imposex-exhibiting female) and site (Hiraiso or Jogashima); and then three composite samples, each containing the respective tissues of 5 individuals by sex and by site, were prepared.

For detection of RXR protein by Western blotting, both normal female and male wild rock shells (approximately 150 individuals) were collected at Hiraiso, in Ibaraki Prefecture, Japan, in August 2004, and reared in a laboratory aquarium with artificial seawater for approximately 1 month for acclimation. The density of rock shells reared in the aquarium was about 150 individuals/155 L; the water temperature,  $23.7 \pm 0.1$  °C; the pH,  $7.82 \pm 0.14$ ; and the salinity,  $33.5 \pm 0.7$  psu, as means  $\pm$  standard deviations. The rock shells were fed on live mussels collected at Hiraiso. The rock shells were dissected to remove the same soft tissues by sex (normal female or male) as mentioned above, and then a composite sample containing the respective tissues from 5 individuals by sex was prepared. The shell heights of males and normal females used here were  $24.9 \pm 1.8$  and  $24.8 \pm 2.4$  mm (mean  $\pm$  standard deviation), respectively.

For immunohistochemical staining for RXR, male and normal female wild rock shells (approximately 100 individuals) were collected at Hiraiso, in Ibaraki Prefecture, Japan, in December 2003 and December 2004. Male and imposex-exhibiting female wild rock shells (approximately

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