Anal Bioanal Chem (2010) 396:597–607
DOI 10.1007/s00216-009-3230-x

ORIGINAL PAPER

Time course of expression of the retinoid X receptor gene and induction of imposex in the rock shell, *Thais clavigera*, exposed to triphenyltin chloride

Toshihiro Horiguchi · Tomohiro Nishikawa · Yasuhiko Ohta · Hiroaki Shiraishi · Masatoshi Morita

Received: 30 July 2009 / Revised: 9 October 2009 / Accepted: 10 October 2009 / Published online: 31 October 2009

Abstract To examine the role of the retinoid X receptor (RXR) in the development of imposex in gastropods, we investigated the time course of expression of the RXR gene in various tissues (ctenidium, ovary or testis, digestive gland, penis-forming area or penis, and head ganglia) of female and male rock shells (*Thais clavigera*) exposed to triphenyltin (TPT) in a flow-through exposure system for 3 months. Accumulations of TPT in tissues were clearly observed in exposed individuals, whereas no accumulation of TPT was observed in the control groups. In females, 3-month exposure to TPT resulted in the development of imposex, and penis lengths in imposex-exhibiting females were significantly longer in small females (shell height <20 mm) than in large females (shell height ≥20 mm). RXR gene expression in the ovary, penis-forming area or penis, and head ganglia of females exposed for 3 months was significantly higher than expression in control females, and the highest RXR gene expression was found in the penis-forming area or penis. Moreover, RXR gene expression in the penis-forming area or penis of each female exposed to TPT seemed to be associated with an increase in penis length. In males, the ratio of penis length to shell height was significantly larger in the exposed groups than in the controls. Although RXR gene expression in males exposed for 3 months was not significantly higher than expression in control males in any tissues, the highest gene expression was observed in the penis of exposed males. These results suggest that RXR plays an important role in the development of male genitalia (i.e., penis and vas deferens) in gastropods, although RXR might also have other physiological functions.

Keywords Imposex · Penis · Vas deferens · Organotin compounds · Rock shell · Retinoid X receptor (RXR)

T. Horiguchi (✉) · T. Nishikawa · H. Shiraishi · M. Morita
Research Center for Environmental Risk,
National Institute for Environmental Studies,
16-2 Onogawa,
Tsukuba, Ibaraki 305-8506, Japan
e-mail: thorigu@nies.go.jp

Y. Ohta
Faculty of Agriculture, Tottori University,
4-101 Koyama-Minami,
Tottori, Tottori 680-8550, Japan

M. Morita
Faculty of Agriculture, Ehime University,
3-5-7 Tarumi,
Matsuyama, Ehime 790-8566, Japan

Introduction

Imposex is an irreversible pseudohermaphroditic condition in which male genital organs, such as the penis and vas deferens, develop in female gastropod molluscs [1, 2]. Imposex is typically induced by very low concentrations (~1 ng/L) of tributyltin (TBT), triphenyltin (TPT), or both; these compounds have been used in antifouling paints for ships and fishing nets since the mid-1960s [1, 3–7]. Reproductive failure may occur in the severe stages of imposex; either because of oviduct blockage by the formation of vasa deferentia or because of ovarian spermatogenesis, and the condition eventually results in population decline or mass extinction [8–11]. More than 150 species of mesogastropods and neogastropods, including the rock shell, *Thais clavigera*, are affected by imposex [12–15]. Imposex among gastropods is considered to be clear evidence for endocrine disruption by environmental pollutants [15, 16].

Four hypotheses regarding the mechanisms by which TBT induces imposex in gastropods have been proposed: (1) an increase in androgen (e.g., testosterone) levels as a result of TBT-mediated inhibition of aromatase [17]; (2) TBT-mediated inhibition of the excretion of androgen sulfate conjugates with consequent increase of androgen levels [18]; (3) TBT interference in the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia [19]; and (4) an increase in the level of an alanine–proline–glycine–tryptophan amide neuropeptide in response to TBT [20]. However, scientific debate about the mechanism continues because none of these hypotheses are sufficient to explain the effects of organotins on the endocrinology of gastropods [21].

T. clavigera has a retinoid X receptor (RXR) that is similar to the RXR receptors in humans and other vertebrates as well as to those in other invertebrates, such as ascidians, insects, gastropod pulmonates, jellyfish, and sponges [22–32]. Nishikawa et al. [22] observed that *T. clavigera* RXR binds both 9-*cis*-retinoic acid (9CRA), which is a natural ligand for human RXRs, and organotins and that a single *in vivo* injection of 9CRA into female rock shells without morphological signs of imposex induces the development of imposex a month later. Thus, Nishikawa et al. proposed that RXR plays an important role in the development of organotin-induced imposex in gastropods.

In previous studies, we investigated RXR gene expression and measured the RXR protein content in various tissues of wild male and female *T. clavigera* by using quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR), Western blotting, and immunohistochemistry with a commercial antibody against human RXR α [33]. We established a polyclonal antibody against the RXR of *T. clavigera* for application to rock shell tissues [34]. Immunoblotting demonstrated that this antibody recognizes *T. clavigera* RXR. In males and imposex-exhibiting females, immunohistochemical staining with the antibody revealed nuclear localization of RXR protein in the epithelial and smooth muscle cells of the vas deferens and in the interstitial and epidermal cells of the penis. These results suggest that the antibody specifically recognizes RXR protein in the tissues of *T. clavigera* and therefore is useful for evaluating RXR protein localization. On the basis of these results, we suggested that RXR may be involved in inducing the development of male-type genitalia (penis and vas deferens) in normal male and organotin-exposed female rock shells.

In the present study, we further elucidated the role of RXR in the development of imposex caused by organotin compounds in gastropods. We used a flow-through exposure system to investigate the effect of TPT exposure on the time course of expression of the RXR gene in various tissues (ctenidium, ovary or testis, digestive gland, penis-forming area or penis, and head ganglia) of female and male *T. clavigera*, as well as the development of imposex in females and changes in penis length in both sexes, over a period of 3 months.

Materials and methods

Collection of rock shells

Female and male rock shells were collected in May 2005 at Aikawa, a reference site on Sado Island in Niigata Prefecture, Japan (38° 01′ 39.75″ N, 138° 14′ 18.48″ E; [TBT]<11 ng/g wet wt, [TPT]<8 ng/g wet wt [6]). The rock shells were reared in a laboratory aquarium for acclimation for approximately 2 months in artificial seawater (Senju Pharmaceutical Co., Osaka, Japan). The rock shells were fed with live mussels (*Septifer virgatus*) collected at Hiraiso, Japan, a site with low levels of organotin contamination in the rock shells (36° 22′ 04.18″ N, 140° 37′ 23.66″ E; [TBT]<10 ng/g wet wt, [TPT]<8 ng/g wet wt) [7].

Flow-through exposure experiments with TPT

Before the experiments, the rock shells were narcotized by exposure to a 72-g/L solution of magnesium chloride hexahydrate to allow the selection of females and males. Female rock shells were identified by the absence of the large penis behind the right tentacle that is observed in the males [6, 7]. The rock shells (200 total; 100 females and 100 males) were evenly divided into two experimental groups by sex, each group consisting of 50 females or 50 males: one group was treated with 500 ng/L of triphenyltin chloride (TPTCl), and the other group was used as a control (treated with acetone and dimethyl sulfoxide [DMSO]) for both sexes. Stock solutions of TPTCl (Tokyo Kasei Kogyo Co., Tokyo, Japan, 98% pure) for the flow-through exposure experiments were prepared in a mixture of acetone and DMSO (1:100) and then diluted with artificial seawater (Tomita Pharmaceutical Co., Ltd., Tokushima, Japan) [35, 36]. The rock shells were kept for 3 months in 2-L glass beakers by experimental groups (control females, exposed females, control males, and exposed males) in flow-through systems of artificial seawater saturated with oxygen (20 L/day) and with acetone–DMSO either with or without TPTCl. Live mussels were used as food. The temperature of the experimental seawater was maintained at 20±1 °C. The characteristics of the artificial seawater during the experimental period (June 13–September 13, 2005) are summarized in Table 1. The rock shells were removed after 1, 2, or 3 months for imposex examination, as well as for an assay of RXR gene expression in various tissues. The survival rates of the

Table 1 Characteristics of artificial seawater during the experimental period (June 13 to September 13, 2005)

	Control females	Control males	TPT-exposed females	TPT-exposed males
Water temp. (°C)	19.8±0.3	19.7±0.3	19.8±0.3	19.8±0.4
pH	7.92±0.06	7.88±0.07	7.96±0.06	7.92±0.07
Salinity (‰)	33.4±1.1	33.2±1.1	33.2±1.2	33.2±1.2

Mean ± standard deviation

rock shells used in all the experimental groups were 100%. The body sizes of the rock shells used in the experiment are shown in Table 2.

Imposex examination

After 1, 2, or 3 months of exposure, 16–18 rock shells were removed from each of the four beakers for imposex examination and for measurement of body size. Shell height and width were measured with a digital caliper (to the nearest 0.01 mm), and body weight was measured with an automatic digital balance (to the nearest 0.1 g). Sex determination and imposex examination were conducted; imposex levels were evaluated along with the accessory reproductive organs, as described by Horiguchi et al. [6]. For males and imposex-exhibiting females, penis length was also measured: the length of the curved penis, which is normal in the male rock shell, was measured with a thread, and the length or diameter of the tiny penis in the initial stage of the development was measured with a digital caliper (to the nearest 0.01 mm). Development of the vas deferens was also evaluated. The following parameters used to evaluate gastropod imposex were calculated for each group [5, 6]: the incidence of imposex equals percentage occurrence of imposex individuals among females used in the experimental group; mean penis length; and vas deferens sequence (VDS) index (an index for the degree of development of the vas deferens in the imposex-exhibiting female; the VDS index for the rock shell is similar to that for the dog-whelk, *Nucella lapillus*, reported by Gibbs et al. [5]).

Then, the ovary or testis, digestive gland, ctenidium, penis-forming area or penis, and head ganglia were removed from the soft tissues of female and male rock shells in each experimental group for assay of RXR gene expression. We prepared three to four composite samples for each group, with each composite sample containing the respective tissues of four individuals. The rest of the soft tissues were used for chemical analysis to determine the tissue concentrations of phenyltins and butyltins.

Preparation of total RNA samples and assay for RXR gene expression in various tissues

Total RNA was extracted from each homogenized sample with an SV Total RNA Isolation System (Promega, Madison, WI, USA), and 100 ng of total RNA was subjected to QRT-PCR in an Access Quick RT-PCR System (Promega), as a reagent for reverse transcription of total RNA and amplification of the resulting cDNA. The certain region in the target gene was amplified with a thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to control the conditions for QRT-PCR. To assay RXR gene expression in various tissues, we employed two primers (forward primer, 5′-GATTCTGGAGGCCGAGATTG-3′; reverse primer, 5′-TGGCTCTTTCTGGGCATCA-3′) and a fluorescence probe (5′-FAM-CGTGGAGCCCAAGATC GACACCTACA-TAMRA-3′; Nippon EGT Co., Toyama, Japan). After being mixed in the Access Quick RT-PCR System, the total RNA, primers, fluorescence probe, and cDNA were incubated at 48 °C for 45 min to synthesize cDNA from the total RNA. Next, the cDNA was amplified under the following conditions: 35 cycles of 94 °C for 15 s, 45.5 °C for 30 s, and 72 °C for 30 s. To normalize RXR gene expression in the respective tissues, expression of 18S ribosomal RNA (18S rRNA) was also assayed in the tissues. To measure the 18S rRNA content of tissue samples, two primers (forward primer, 5′-GGGTTCTGCCCGTCCTTT-3′; reverse primer, 5′-CCGCTACCCGTTGCTAACA-3′) and a fluorescence probe (5′-FAM-TGACGATGGTACGT GATCTGCCTACCA -TAMRA -3′; Nippon EGT Co.) were employed. We normalized the RXR gene expression in each tissue by dividing the value for the RXR gene content by the value for the 18S rRNA content [37, 38].

Table 2 Body sizes of female and male rock shells used in the flow-through exposure experiments (June 13 to September 13, 2005)

	Control females	Control males	TPT-exposed females	TPT-exposed males
Shell height (mm)	18.8±3.0	20.6±2.4	18.9±3.1	18.6±2.6
Shell width (mm)	13.2±2.3	14.3±2.7	13.5±2.2	13.1±2.0
Shell weight (g)	1.50±0.83	1.93±0.65	1.52±0.79	1.42±0.70

Mean ± standard deviation

Chemical analysis of organotin compounds in tissues of rock shells exposed to TPT

Chemical analysis of organotin compounds (TPT, TBT, and their metabolites) in the rest of the soft tissues was conducted according to the methods of Horiguchi et al. [6]. Briefly, tissues were extracted with 0.1% tropolone–benzene and 1 N hydrobromic acid–ethanol by ultrasonication, derivatized with propylmagnesium bromide, purified by silica gel column chromatography, and quantified by gas chromatography with flame photometric detection. The detection limit of the instrument was 50 pg, corresponding to 15.2 to 34.5 ng/g wet tissue sample. Certified reference material for TBT and TPT analysis (Japanese sea bass, *Lateolabrax japonicus*, prepared by the National Institute for Environmental Studies; NIES CRM no. 11) was used for quality assurance and quality control.

Data analysis and statistics

The statistical significance of any difference in imposex levels compared with the control group was tested. The statistical significance of the incidence of imposex was determined by Fisher's *t* test, and one-factor analysis of variance (ANOVA) was used for penis length and VDS index [20].

For RXR gene expression in each tissue, we evaluated the statistical significance of differences between the TPT-exposed groups and the control groups by one-factor ANOVA, assuming that RXR gene expression had an equal variance and that the expression followed a normal distribution.

Results

Accumulation of TPT and induction of imposex in the rock shell

The levels of phenyltins and butyltins were below the detection limit in tissues of both female and male rock shells before exposure and in the control animals. In contrast, marked accumulation of TPT in tissue was observed in both females and males exposed to TPT, even after 1 month of exposure (Fig. 1).

The incidence of imposex was significantly higher in the exposed females than in the control females ($p<0.01$ after 1 month of exposure; $p<0.001$ after 2 and 3 months of exposure; Fig. 2). The incidence of imposex in females exposed to TPT was positively correlated with the tissue concentrations of TPT (Figs. 1a and 2). Female penis length gradually but significantly increased up to 2 months of exposure ($p<0.01$ after 1 month of exposure; $p<0.001$ after 2 months of exposure; Fig. 3) and markedly increased after 3 months of exposure, although a large standard deviation was observed at 3 months ($p<0.001$; Fig. 3). The VDS index also increased significantly ($p<0.01$ after 1 month of exposure; $p<0.001$ after 2 and 3 months of exposure; Table 3).

Interestingly, the physiological response to induction and promotion of the development of imposex varied between shell height classes: imposex was already significantly induced in small females (shell height <20 mm) after 1 month of exposure ($p<0.01$ after 1 month of exposure; $p<0.001$ after 2 and 3 months of exposure), whereas imposex was not significantly induced in large females (shell height ≥20 mm) up to 2 months of exposure ($p>0.05$) and significantly induced after 3 months of exposure ($p<0.01$). The degree of increase in penis length was much more remarkable in small females ($p<0.05$ after 1 month of exposure; $p<0.001$ after 2 and 3 months of exposure; Fig. 4b) than in large females ($p<0.01$ after 3 months of exposure; Fig. 4a). The increase in the VDS index was similar to that in penis length ($p<0.05$ after 1 month of exposure, and $p<0.001$ after 2 and 3 months of exposure for small females; $p<0.01$ after 3 months of exposure for large females).

Penis length in males increased slightly over 3 months in exposed males, whereas penis length decreased substantially in the control males (Fig. 5).

RXR gene expression

RXR gene expression was measured in the testis or ovary, digestive gland, ctenidium, penis or penis-forming area, and head ganglia of female and male rock shells in each experimental group (Figs. 6 and 7). RXR gene expression in female rock shells exposed to TPT was highest in the penis-forming area or the penis, although it was statistically insignificant because of the large standard deviation (Fig. 6d). Significantly greater expression of the RXR gene was observed in the ovary and head ganglia of females exposed to TPT than in control females, although the expression values were much smaller than those observed in the penis-forming area or the penis ($p<0.01$ after 1 and 2 months of exposure and $p<0.05$ after 3 months of exposure in ovary; $p<0.01$ after 1 and 3 months of exposure in the head ganglia; Fig. 6b and e).

Comparison of the values of RXR gene expression in three composite samples of exposed females with the respective values for penis length revealed that RXR gene expression seemed to be associated with an increase in penis length: gene expression of RXR was high in the sample of exposed females with long penises (Fig. 8).

No significant increase in expression of the RXR gene was observed in any tissue of males exposed to TPT relative to expression in the control males, although the expression values in the penis were higher than in any other tissue or organ examined (Fig. 7).

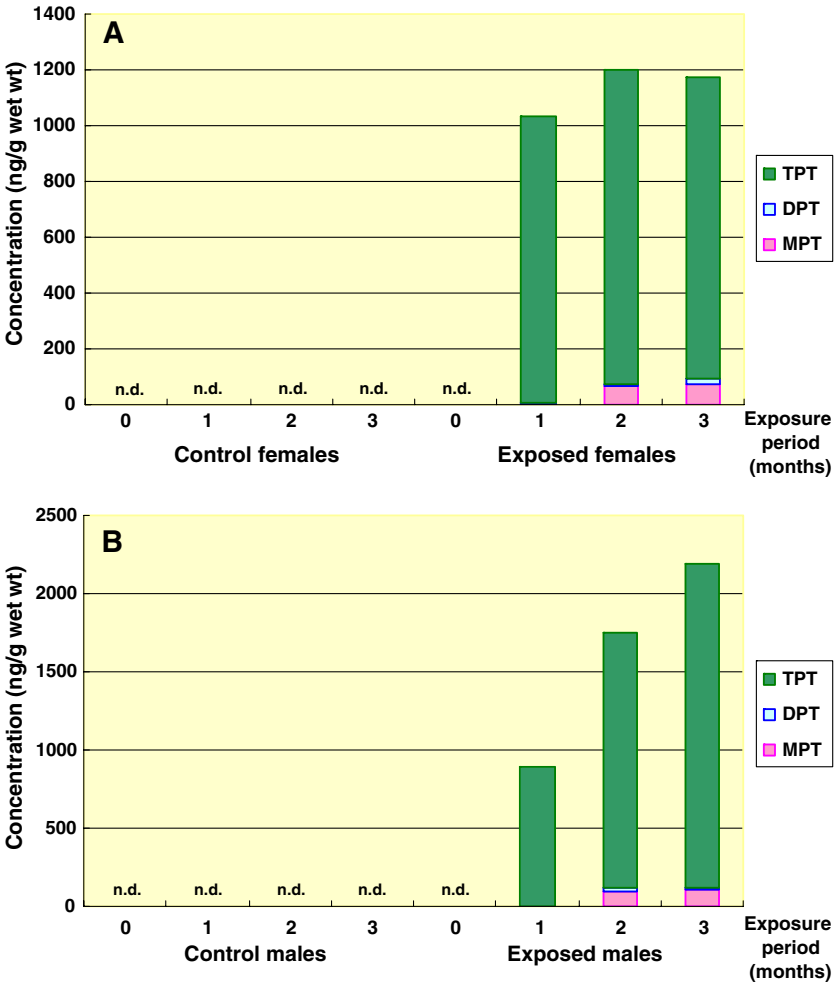

Fig. 1 Accumulation of phenyltin compounds in tissues of **a** female and **b** male rock shells exposed to 500 ng/L of triphenyltin chloride (TPTCl) for 3 months in a flow-through system. *TPT* triphenyltin, *DPT* diphenyltin, *MPT* monophenyltin, *n.d.* not detected

Discussion

Accumulation of TPT in the rock shell

The soft tissues remaining after removal of the ovary or testis, digestive gland, ctenidium, penis-forming area or penis, and head ganglia were used for chemical analysis to determine tissue concentrations of phenyltin compounds. Therefore, the mean overall tissue TPT concentration were probably much higher than the measured values, because TPT is known to accumulate more in the ovary or testis, digestive gland, ctenidium, and head ganglia [11, 39–43].

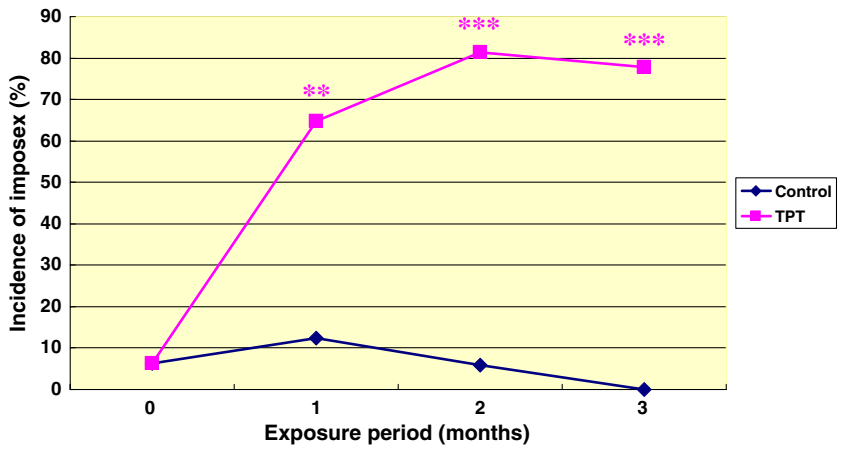

Fig. 2 Temporal change of incidence of imposex in female rock shells exposed to 500 ng/L of triphenyltin chloride (TPTCl) for 3 months in a flow-through system. *TPT* triphenyltin; $^{**}p<0.01$; $^{***}p<0.001$

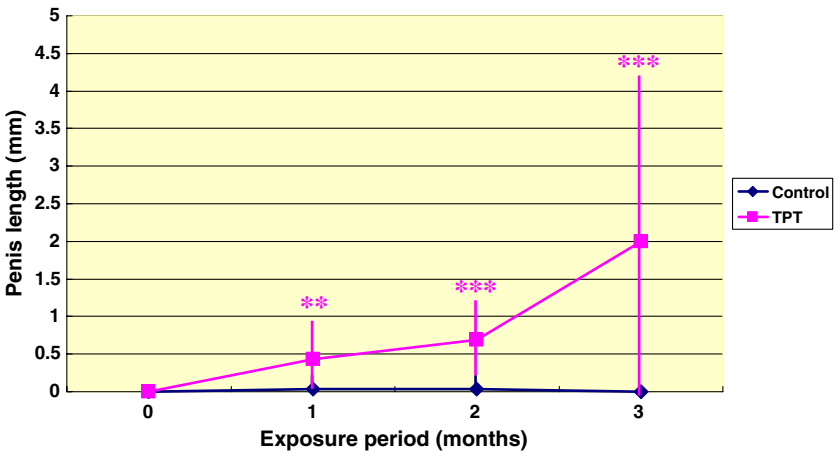

Fig. 3 Temporal change of average penis length in female rock shells exposed to 500 ng/L of triphenyltin chloride (TPTCl) for 3 months in a flow-through system. *Vertical bars* represent standard deviations. *TPT* triphenyltin, $^{**}p<0.01$; $^{***}p<0.001$

In the ivory shell (*Babylonia japonica*), the highest concentrations of TPT are detected in the ovaries of females and in the digestive glands of males; on the basis of the total body burden of TPT, approximately three fourths and greater than half of all TPT accumulates in the digestive glands of males and females of *B. japonica*, respectively [11]. The second-highest tissue burden of TPT is observed in the gonads of both males and females, followed by the muscle, ctenidium, and heart in males and the muscle, oviduct, and head in females of *B. japonica* [11]. Accumulation of organotin compounds in the head ganglia of *T. clavigera* [41], *N. lapillus* [38], and *Buccinum undatum* [43] has also been reported. Although the concentrations of TBT and TPT in the head ganglia are quite high in *T. clavigera*, the total tissue burden of those organotins is low because of the relatively small amount of ganglion tissue in that species [41]; this may also be the case in *B. japonica* [11]. Similar concentrations of TBT and TPT to *B. japonica* have been detected in the ganglia of *B. undatum* [43].

Our chemical analysis showed that the tissue concentrations of TPT were higher in males than in females (Fig. 1). However, this result does not necessarily mean that TPT accumulated more in males than in females, again because the soft tissues remaining after removal of the ovary or testis, digestive gland, ctenidium, penis-forming area or penis, and head ganglia were used to determine tissue concentrations of the phenyltin compounds. In fact, TPT and TBT concentrations in whole soft tissues of imposex-exhibiting female *T. clavigera* specimens were generally higher than in male ones, possibly owing to greater accumulation of TBT and TPT in the female reproductive organs, such as aborted egg capsule mass in the capsule gland, than in the male reproductive organs [6].

TPT was a predominantly accumulated phenyltin species in both females and males of *T. clavigera*, suggesting that the metabolism of TPT is low in *T. clavigera*. The ability to metabolize TBT differs among species but the ability to metabolize TPT appears to be generally low in many kinds of organisms [3, 11, 39–42, 44–47]. The biological and ecological half-lives of TBT and TPT are estimated to be 22 and 347 days, respectively, in *T. clavigera* [48].

Table 3 Vas deferens sequence index (VDS) in female rock shells during 3-month exposure to TPT in a flow-through system

VDS	Initial	1 month	2 months	3 months
Control	0.06±0.25	0.13±0.34	0.06±0.24	0.00±0.00
TPT-treated	0.06±0.25	0.76±0.75*	1.06±0.85**	1.82±1.70**

Mean ± standard deviation

*$p<0.01$

**$p<0.001$

Induction of imposex in the rock shell and RXR gene expression after exposure to TPT

Our results strongly suggest that exposure to TPT led to significantly increased expression of the RXR gene, which resulted in induction and promotion of the development of imposex in the rock shell (Figs. 1, 2, 3, 4, 6, and 8). The values of RXR gene expression in three composite samples of the penis-forming area or the penis in exposed females appeared to be positively correlated with penis length (Fig. 8). These results also support our hypothesis that RXR plays important roles in the differentiation or proliferation (or both) of certain cells responsible for the development of imposex conditions in the penis-forming area of females, in a manner that is apparently similarly to that in males [33, 34, 49].

In exposed female rock shells, the induction of imposex and the increase in penis length were clearly observed after 1

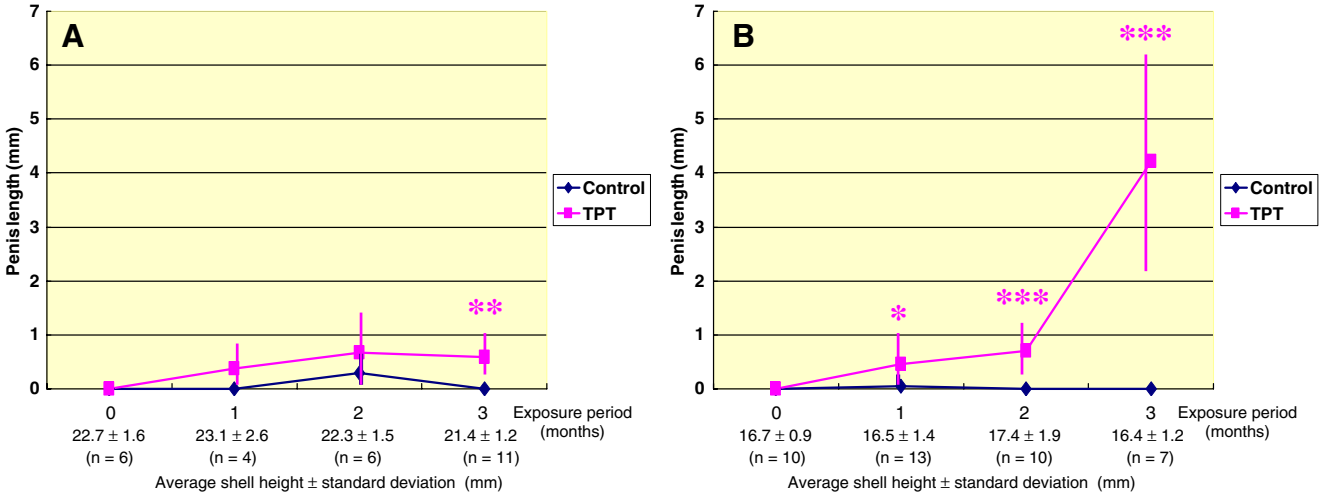

Fig. 4 Temporal change of average penis length in **a** large (shell height ≥20 mm) and **b** small (shell height <20 mm) female rock shells exposed to 500 ng/L of triphenyltin chloride (TPTCl) for 3 months in a flow-through system. *Vertical bars* represent standard deviations. *TPT* triphenyltin; *p<0.05; **p<0.01; ***p<0.001

and 2–3 months of exposure, respectively (Figs. 2, 3, and 4). In previously reported results for injection experiments, the induction of imposex and penis growth in female rock shells injected with TPT solution required 2 weeks and 1 month, respectively, where approximately 500–600 ng TPT/g wet tissue were detected in females (20.5±2.8 mm as mean shell height ± standard deviation) at 2 and 30 days after the injections [7]. The differences between the times required for the development of imposex conditions may be due to differences in the rate of accumulation of TPT to a level that exceeds the threshold concentration for increased RXR expression in target organs/cells between the two different exposure methods (i.e., injection and the flow-through exposure experiments). However, the threshold concentration of TPT for RXR induction to initiate the development of imposex is unknown. The results also suggest that some time is required for differentiation of certain epithelial cells (i.e., stem cells) into penis and vas deferens cells and the subsequent proliferation of those cells for growth of the penis and development of the vas deferens. It is also possible that TPT binds relatively weakly, which may be why a clear increase in penis length in female rock shells required 3 months of exposure to TPT.

Our observation that small (shell height <20 mm) females, which may be young, seemed more sensitive to TPT than large (shell height ≥20 m) females, which may be old, does not contradict field observations of strong expression of the RXR gene in the tiny penis of a small (possibly young) female rock shell in Hiraiso, Japan [33]. Larvae and juveniles are generally more sensitive to pollutants, including organotins, than are adults [50]. Further studies are necessary to determine the origin of differences in sensitivity to pollutants at different life stages and ages.

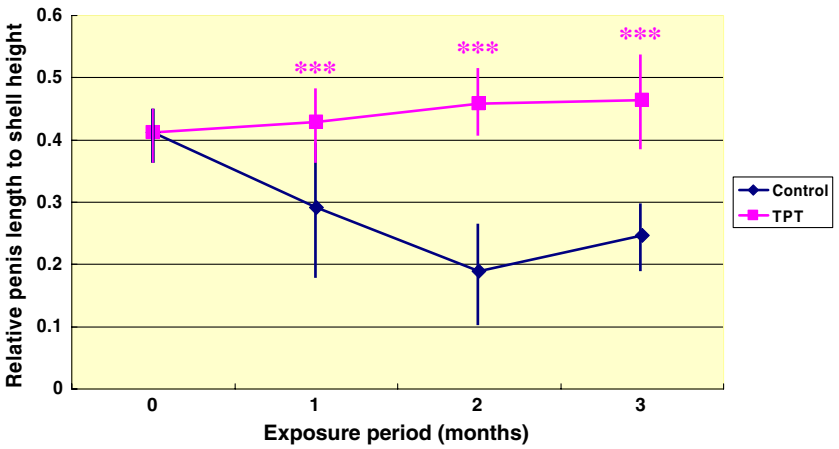

Fig. 5 Temporal change of ratio of average penis length to shell height in male rock shells exposed to 500 ng/L of triphenyltin chloride (TPTCl) for 3 months in a flow-through system. *Vertical bars* represent standard deviations. *TPT* triphenyltin; ***p<0.001

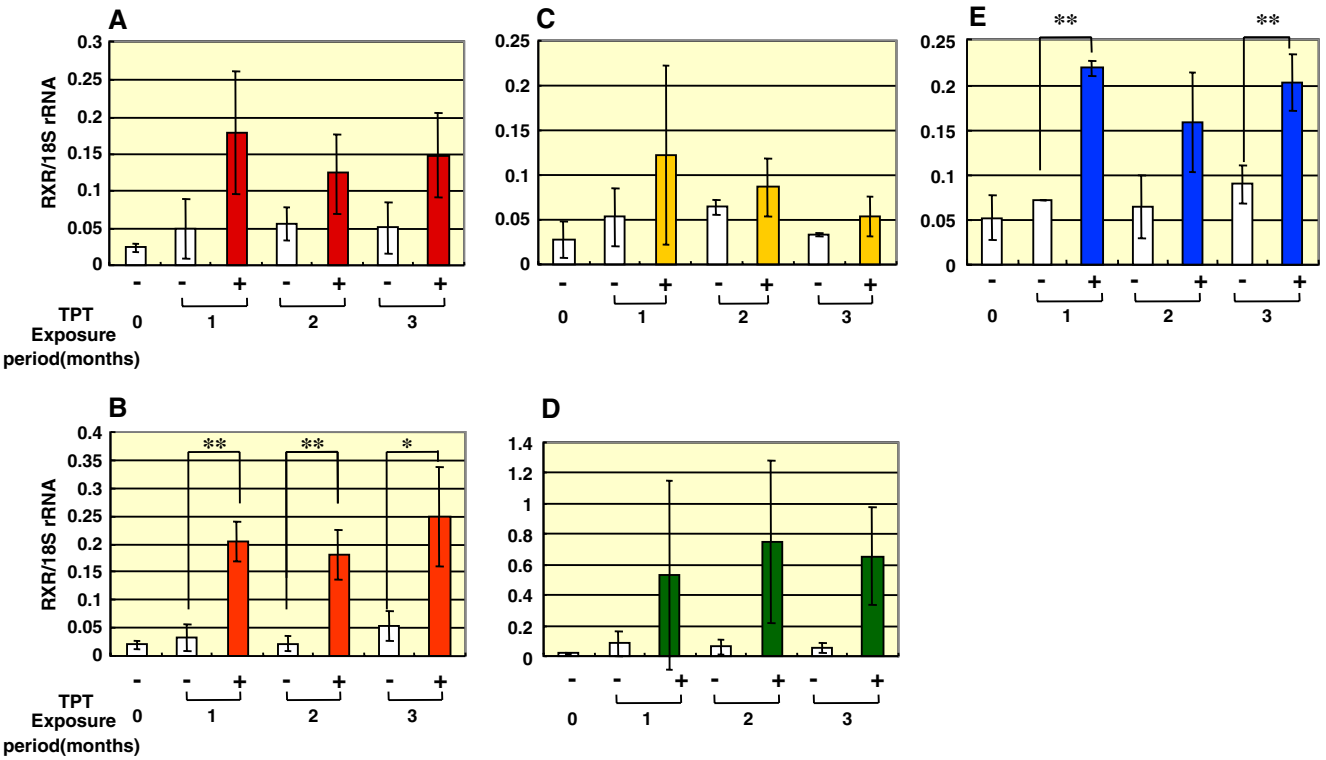

Fig. 6 Normalized RXR gene expression in **a** ctenidium, **b** ovary, **c** digestive gland, **d** penis-forming area or penis, and **e** head ganglia of female rock shells exposed to 500 ng/L of triphenyltin chloride (TPTCl) for 3 months in a flow-through system. *minus sign* control females; *plus sign* TPT-exposed females; *TPT* triphenyltin; *$p<0.05$; **$p<0.01$

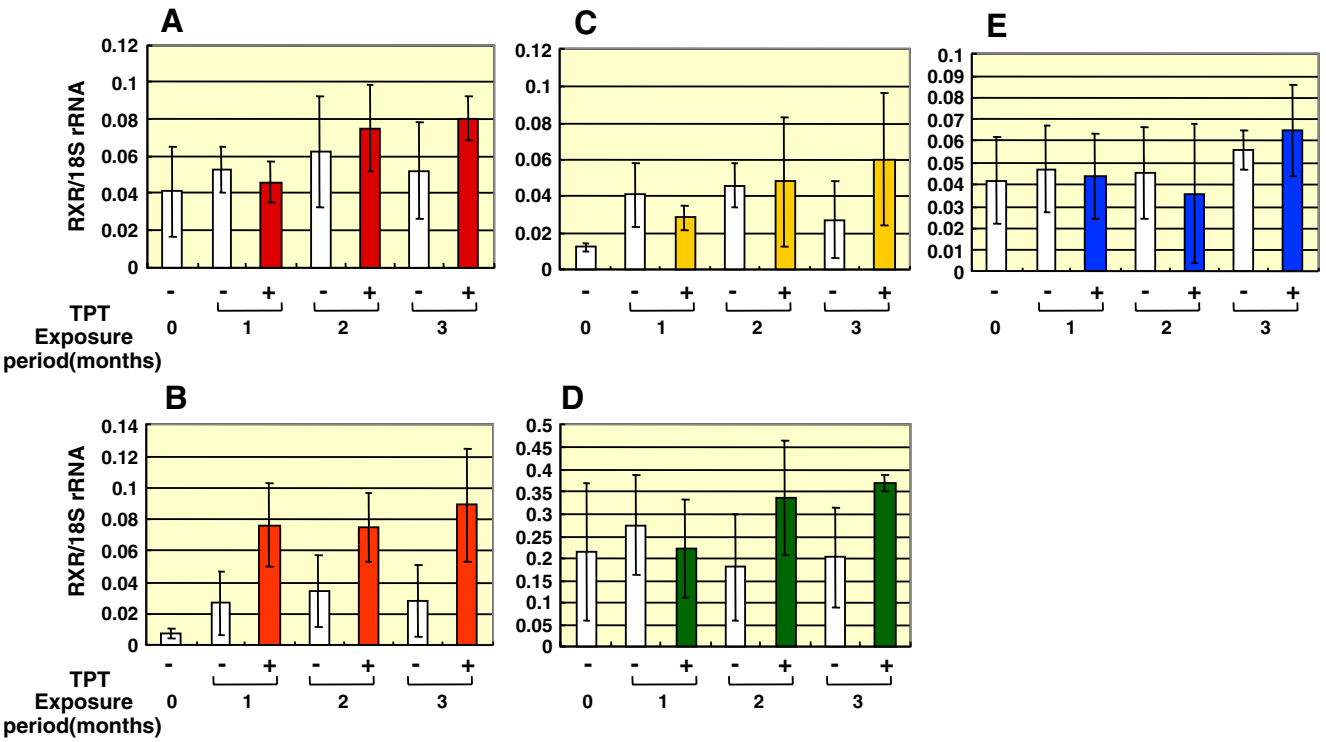

Fig. 7 Normalized RXR gene expression in **a** ctenidium, **b** testis, **c** digestive gland, **d** penis, and **e** head ganglia of male rock shells exposed to 500 ng/L of triphenyltin chloride (TPTCl) for 3 months in a flow-through system. *minus sign* control males, *plus sign* TPT-exposed males; *TPT* triphenyltin

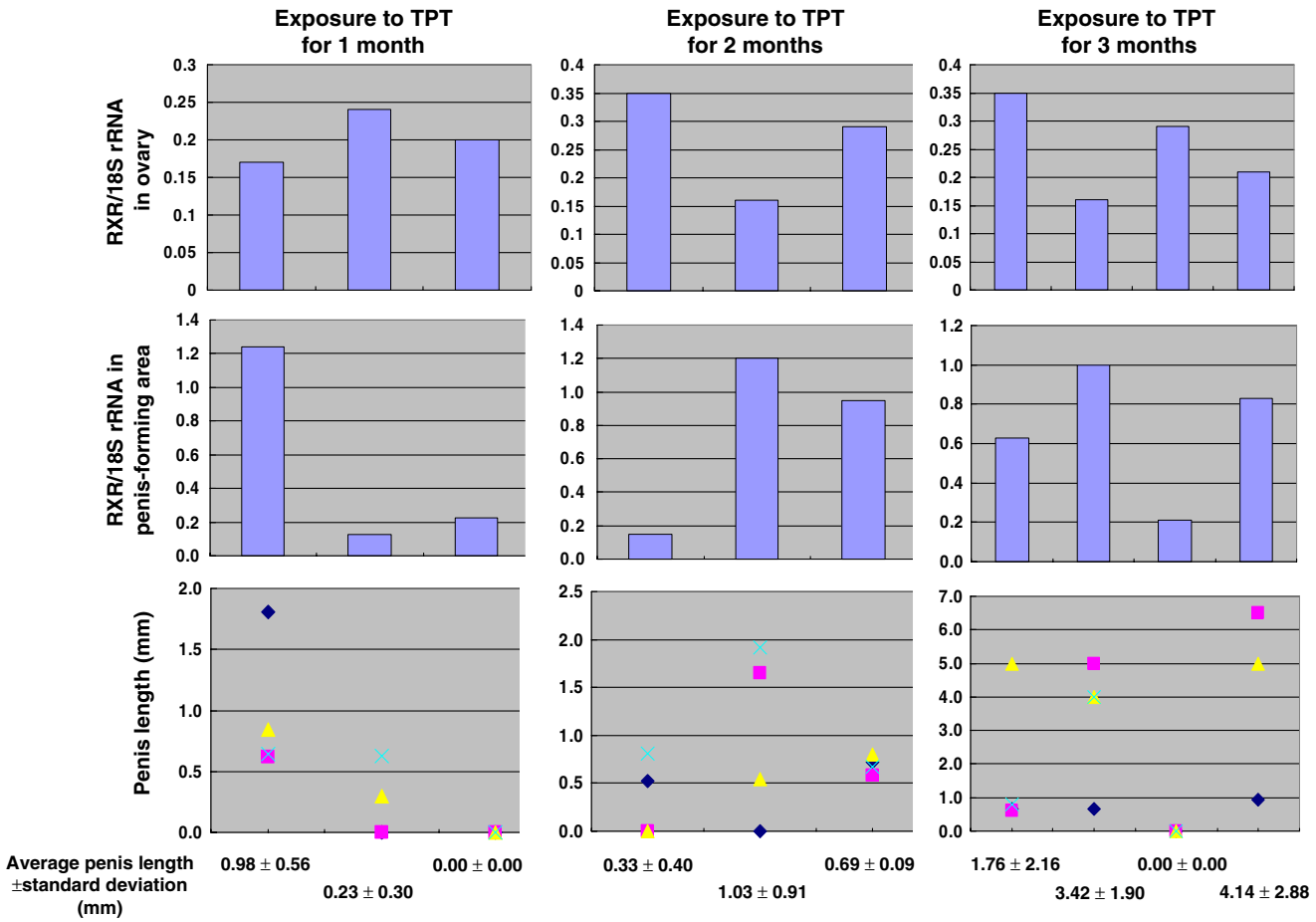

Fig. 8 Relationships between average penis length and RXR gene expression in penis-forming area/penis and ovary of female rock shells exposed to 500 ng/L of triphenyltin chloride (TPTCl) for 3 months in a flow-through system. *TPT* triphenyltin. *Bars in the upper and middle figures* represent normalized RXR gene expression in ovary and penis-forming area/penis of females exposed to TPTCl, respectively. *Dots or symbols in the bottom figure* represent measured values of penis length of each female in the respective composite samples. RXR gene expression in the penis-forming area or penis of each female exposed to TPT seems to be associated with an increase in penis length; however, that in ovary does not

No seasonal changes in penis length were observed in males and imposex-exhibiting females collected in the mid-1980s and early 1990s at sites contaminated by organotins such as TBT and TPT [3, 35]. However, clear seasonal changes of penis length were observed in male rock shells collected in a pristine location at Hiraiso, Japan, in the 2000s; a drastic decrease of penis length was observed in males collected in late August, just after the end of the spawning season; this decrease seemed to be the result of cell apoptosis [51]. These results suggest that there may be a seasonal change in penis length in males, but that organotin contamination stimulates or up-regulates the expression of RXR in gastropod tissues; continuing interaction between organotins and RXR may thus be responsible for the undiminished penis length in both males and imposex-exhibiting females, possibly by promotion of penis growth. This interaction may also inhibit the regression of the penis that occurs as a result of unknown retrogressive factors initiated by certain natural, seasonal cues [19]. If this speculation is accurate, our current results for male rock shells (Fig. 5) could be explained by the following scenario: penis length in the control males decreased during the experimental period (from June to September) owing to unknown retrogressive factors initiated by seasonal cues, whereas penis length in the exposed males remained stable or increased slightly owing to limited stimulus by the up-regulated expression of RXR in tissue due to exposure to TPT. In males, expression of the RXR gene was higher in the penis than in any other tissue of organ examined, although the expression in the penis of the exposed males was insignificantly higher than in the penis of the control males (Fig. 7). These results also suggest that TPT has a positive effect on penis growth in the male rock shell, possibly through interaction with RXR. Verification of this hypothesis will require additional study.

Mode of action of TBT and TPT in the development of imposex in gastropods

Although 9CRA is known to be the natural ligand for RXRs in vertebrates [26, 29, 30, 52], whether the same is true in *T. clavigera* RXR is not clear, because 9CRA is difficult to detect *in vivo* [53]. The natural ligand for rock shell RXR may be some compound other than 9CRA. Identification of the natural ligand for *T. clavigera* RXR is required for further analysis of the mechanism of imposex induction by organotins in gastropods. Dmetrichuk et al. [54] recently detected all-*trans* retinoic acid (ATRA) and 9CRA in the central nervous systems of adults of the pulmonate gastropod *Lymnaea stagnalis* by high-performance liquid chromatography–mass spectrometry. Because ATRA and 9CRA were detected in the tissues of *L. stagnalis*, this species likely also has metabolic enzymes to synthesize or transform retinoic acids (RAs). The presence of 9CRA should be investigated in *T. clavigera*. Whether the rock shell can synthesize 9CRA, ATRA, or both from β carotene must also be determined; there have been no reports describing the genes encoding the retinoic acid receptor or the enzymes involved in the synthesis and metabolism of RAs (e.g., Raldh2, Cyp26) in invertebrates, except in the Prochordata (e.g., ascidians and amphioxus) [55, 56]. Because docosahexaenoic acid also acts as a ligand for RXR in the brain of the fetal mouse [57], the possibility that docosahexaenoic acid is a natural ligand for *T. clavigera* RXR should also be examined.

In addition to identifying the natural ligand for *T. clavigera* RXR, we must also examine the binding and activation properties of the ligand with respect to RXR, determine whether RXR forms homodimers or homotetramers or heterodimers with other nuclear receptors, and determine the physiological and biochemical responses downstream of the RXR signaling pathway if we are to elucidate the mechanisms of RXR-mediated transcription regulation [34]. RXR may also have multiple physiological functions in gastropods [34]. Such studies would improve our understanding of the mechanism of induction of imposex by organotin compounds such as TBT and TPT in gastropods, as well as the differentiation, growth, and formation of male-type genitalia in normal males.

Overall, our results are consistent with previous results on the interaction between RXR and the development of imposex in gastropods [22, 33, 34, 49, 58]: RXR may play an important role in the differentiation or growth (or both) of the penis and vas deferens in male and imposex-exhibiting female gastropods. However, males may also have factors that cause retrogression in penis length. Further studies are needed to clarify the physiological factors related to the differentiation and growth of the penis and vas deferens in male and imposex-exhibiting female gastropods, as well as regression of the penis in males, and to elucidate the molecular mechanism of initiation and promotion of the development of imposex in gastropods by TBT and TPT.

Acknowledgment This work was supported partly by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Scientific Research [B], no. 17380121).

References

1. Bryan GW, Gibbs PE, Hummerstone LG, Burt GR (1986) J Mar Biol Assoc UK 66:611–640
2. Smith BS (1971) Proc Malacol Soc Lond 39:377–378
3. Bryan GW, Gibbs PE, Burt GR, Hummerstone LG (1987) J Mar Biol Assoc UK 67:525–544
4. Bryan GW, Gibbs PE, Burt GR (1988) J Mar Biol Assoc UK 68:733–744
5. Gibbs PE, Bryan GW, Pascoe PL, Burt GR (1987) J Mar Biol Assoc UK 67:507–523
6. Horiguchi T, Shiraishi H, Shimizu M, Morita M (1994) J Mar Biol Assoc UK 74:651–669
7. Horiguchi T, Shiraishi H, Shimizu M, Morita M (1997) Environ Pollut 95:85–91
8. Gibbs PE, Bryan GW (1986) J Mar Biol Assoc UK 66:767–777
9. Gibbs PE, Pascoe PL, Burt GR (1988) J Mar Biol Assoc UK 68:715–731
10. Gibbs PE, Bryan GW, Pascoe PL, Burt GR (1990) J Mar Biol Assoc UK 70:639–656
11. Horiguchi T, Kojima M, Hamada F, Kajikawa A, Shiraishi H, Morita M, Shimizu M (2006) Environ Health Perspect 114(Suppl 1):13–19
12. Fioroni P, Oehlmann J, Stroben E (1991) Zool Anz 226:1–26
13. Horiguchi T, Shiraishi H, Shimizu M, Morita M (1997) Appl Organomet Chem 11:451–455
14. Horiguchi T (2000) In: Kawai S, Koyama J (eds) Problems of endocrine disruptors in fisheries environment. [in Japanese] Koseisha-Koseikaku, Tokyo
15. Matthiessen P, Reynoldson T, Billinghurst Z, Brassard DW, Cameron P, Chandler GT, Davies IM, Horiguchi T, Mount DR, Oehlmann J, Pottinger TG, Sibley PK, Thompson A, Vethaak AD (1999) In: de Fur PL, Ingersoll C, Tattersfield L (eds) Endocrine disruption in invertebrates: endocrinology, testing, and assessment. SETAC, Florida
16. Matthiessen P, Gibbs PE (1998) Environ Toxicol Chem 17:37–43
17. Bettin C, Oehlmann J, Stroben E (1996) Helgol Meeresunters 50:299–317
18. Ronis MJJ, Mason AZ (1996) Mar Environ Res 42:161–166
19. Féral C, Le Gall S (1983) In: Lever J, Boer HH (eds) Molluscan neuro-endocrinology. North-Holland, Amsterdam
20. Oberdörster E, McClellan-Green P (2000) Peptides 21:1323–1330
21. Horiguchi T (2009) In: Arai T, Harino H, Ohji M, Langston WJ (eds) Ecotoxicology of antifouling biocides. Springer, Tokyo
22. Nishikawa J, Mamiya S, Kanayama T, Nishikawa T, Shiraishi F, Horiguchi T (2004) Environ Sci Technol 38:6271–6276
23. Bouton D, Escriva H, de Mendonca RL, Glineur C, Bertin B, Noël C, Robinson-Rechavi M, de Groot A, Cornette J, Laudet V, Pierce RJ (2005) J Mol Endocrinol 34:567–582
24. Devine C, Hinman VF, Degnan BM (2002) Int J Biol 46:687–692
25. Freebern WJ, Osman A, Niles EG, Christen L, LoVerde PT (1999) J Biol Chem 247:4577–4585
26. Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C (1992) Cell 68:397–406

27. Kamimura M, Fujiwara S, Kawamura K, Yubisui T (2000) Dev Growth Differ 42:1–8
28. Kostrouch Z, Kostrouchova M, Love W, Jannini E, Piatigorsky J, Rall JE (1998) Proc Natl Acad Sci USA 95:13442–13447
29. Mangelsdorf DJ, Evans RM (1995) Cell 83:841–850
30. Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A, Evans RM (1992) Genes Dev 6:329–344
31. Nagatomo K, Ishibashi T, Satou Y, Satoh N, Fujiwara S (2003) Mech Dev 120:363–372
32. Wiens M, Batel R, Korzhev M, Muller WE (2003) J Exp Biol 206:3261–3271
33. Horiguchi T, Nishikawa T, Ohta Y, Shiraishi H, Morita M (2007) Aquat Toxicol 84:379–388
34. Horiguchi T, Urushitani H, Ohta Y, Iguchi T, Shiraishi H (2009) Ecotoxicol under review
35. Horiguchi T (1993) Imposex Caused by Organotin Compounds in Marine Gastropods from Japan [PhD Thesis, in Japanese]. Bunkyo-ku, Tokyo: The University of Tokyo
36. Yamada H, Takayanagi K (1992) Water Res 26:1589–1595
37. Higuchi R, Fockler C, Dollinger G, Watson R (1993) Biotechnol 11:1026–1030
38. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Genome Res 6:986–994
39. Bryan GW, Bright DA, Hummerstone LG, Burt GR (1993) J Mar Biol Assoc UK 73:889–912
40. Horiguchi T, Takiguchi N, Cho HS, Kojima M, Kaya M, Shiraishi H, Morita M, Hirose H, Shimizu M (2000) Mar Environ Res 50:223–229
41. Horiguchi T, Shiraishi H, Morita M (2003) [Abstract] SETAC 24th Annual Meeting Abstract Book, p 290
42. Horiguchi T, Kojima M, Takiguchi N, Kaya M, Shiraishi H, Morita M (2005) Mar Pollut Bull 51:817–822
43. Mensink B, Boon JP, ten Hallers-Tjabbes CC, van Hattum B, Koeman JH (1997) Environ Technol 18:1235–1245
44. Bock R (1981) In: Gunter FA, Gunter JD (eds) Residue reviews, vol 79. Springer, New York
45. Lee RF (1985) Mar Environ Res 17:145–148
46. Matsuda R, Suzuki T, Saito Y (1993) J Agric Food Chem 41:489–495
47. Suzuki T, Kondo K, Uchiyama M, Murayama M (1999) J Agric Food Chem 47:4791–4798
48. Horiguchi T, Shiraishi H, Shimizu M, Yamazaki S, Morita M (1995) Mar Pollut Bull 31:402–405
49. Horiguchi T, Ohta Y, Nishikawa T, Shiraishi F, Shiraishi H, Morita M (2008) Cell Biol Toxicol 24:553–562
50. Fent K (1996) Crit Rev Toxicol 26:1–117
51. Horiguchi T, Nishikawa T, Ohta Y, Shiraishi H (2008) [Abstract] SETAC Europe 18th Annual Meeting Abstract Book, p 150
52. Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen C, Rosenberger M, Lovey A (1992) Nature 355:359–361
53. Horton C, Maden M (1995) Dev Dyn 202:312–323
54. Dmetrichuk JM, Carlone RL, Jones TRB, Vesprini ND, Spencer GE (2008) J Neurosci 28:13014–13024
55. Escriva H, Safi R, Hanni C, Langlois MC, Saumitou-Laprade P, Stehelin D, Capron A, Pierce R, Laudet V (1997) Proc Natl Acad Sci USA 94:6803–6808
56. Nagatomo K, Fujiwara S (2003) Gene Expr Patterns 3:273–277
57. de Urquiza AM, Liu S, Sjoberg M, Zetterstrom RH, Griffiths W, Sjovall J, Perlmann T (2000) Science 290:2140–2144
58. Castro LFC, Lima D, Machado A, Melo C, Hiromori Y, Nishikawa J, Nakanishi T, Reis-Henriques MA, Santos MM (2007) Aquat Toxicol 85:57–66