Chapter 9
Mode of Action of Organotins to Induce the Development of Imposex in Gastropods, Focusing on Steroid and the Retinoid X Receptor Activation Hypotheses

Toshihiro Horiguchi

Abstract Basic knowledge of endocrinology or reproductive physiology of prosobranch gastropods is reviewed, focusing on vertebrate-type steroids as possible sex hormones in gastropods. Major points of the view for criticism are steroid-producing cells, enzymes to synthesize and/or metabolize steroids, and functional receptors for steroids. Mechanism of induction and promotion of the development of imposex is also reviewed, regarding six hypotheses proposed as the mechanism by which organotins, such as TBT and TPhT, induce the development of imposex in gastropods: (1) an increase in androgen (e.g., testosterone) levels as a result of TBT-mediated inhibition of aromatase; (2) an increase in testosterone levels owing to the inhibition of acyl CoA-steroid acyltransferase; (3) TBT-mediated inhibition of the excretion of androgen sulfate conjugates, with a consequent increase in androgen levels; (4) TBT interference with the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia; (5) an increase in the level of an alanine-proline-glycine-tryptophan amide (APGWamide) neuropeptide in response to TBT; and (6) activation of the retinoid X receptor (RXR). The latest information about nuclear receptors other than RXR in gastropods, namely, retinoic acid receptor (RAR) and peroxisome proliferator-activated receptor (PPAR), is also described.

Keywords Aromatase • Alanine-proline-glycine-tryptophan amide (APGWamide) • Enzymes to synthesize steroids • Functional receptors for steroids • Retinoid X receptor (RXR) • Sex hormones • Steroid-producing cells • Tributyltin (TBT) • Triphenyltin (TPhT) • Vertebrate-type steroids

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Abbreviations

PPAR	peroxisome proliferator-activated receptor
RAR	retinoic acid receptor
RXR	retinoid X receptor
TBT	tributyltin
TPhT	triphenyltin

9.1 Introduction

In the former part of this chapter, basic knowledge of endocrinology or reproductive physiology of prosobranch gastropods is reviewed, in terms of vertebrate-type steroids. Because neuropeptides of mollusks, including gastropods, are reviewed in the previous chapter (Chap. 8), I will critically review existed knowledge of vertebrate-type steroids detected in gastropods, focusing on the possibility of vertebrate-type steroids to be sex hormones in gastropods. Major points of the view for criticism are steroid-producing cells, enzymes to synthesize and/or metabolize steroids, and functional receptors for steroids. The latest information about genes coding vertebrate-type steroid hormone receptors in gastropods is also described.

In the latter part of this chapter, mechanism of induction and promotion of the development of imposex is reviewed: regarding the mechanism by which organotins, such as TBT and TPhT, induce the development of imposex in gastropods, six hypotheses have been proposed:

1. An increase in androgen (e.g., testosterone) levels as a result of TBT-mediated inhibition of aromatase (Bettin et al. 1996)
2. An increase in testosterone levels owing to the inhibition of acyl CoA-steroid acyltransferase (Gooding et al. 2003; Sternberg and LeBlanc 2006)
3. TBT-mediated inhibition of the excretion of androgen sulfate conjugates, with a consequent increase in androgen levels (Ronis and Mason 1996)
4. TBT interference with the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia (Féral and Le Gall 1983)
5. An increase in the level of an alanine-proline-glycine-tryptophan amide (APGWamide) neuropeptide in response to TBT (Oberdörster and McClellan-Green 2000)
6. Activation of the retinoid X receptor (RXR) (Nishikawa et al. 2004)

Although scientific debate is still continuing, there are several papers in which a hypothesis of activation of RXR is supported (Castro et al. 2007; Horiguchi et al. 2007, 2008a, 2010a, b; Sternberg et al. 2008; Urushitani et al. 2011). The latest information about nuclear receptors other than RXR in gastropods, namely, retinoic acid receptor (RAR) and peroxisome proliferator-activated receptor (PPAR), is also described (see below).

9.2 A Critical Review on Steroid Hormones in Gastropods

Because sex steroid hormones, such as testosterone and 17β-estradiol, play physiologically important roles in the development of sex organs and the maturation of gonads (i.e., oogenesis and spermatogenesis) in vertebrates, it has been hypothesized that similar sex steroid hormones might also regulate the reproduction of invertebrates, such as gastropods (LeBlanc et al. 1999). After the removal of the hermaphroditic organ, oogenesis and spermatogenesis were observed, respectively, in the gonads of 17β-estradiol-treated females and testosterone-treated males of the slug *Limax marginatus*; egg laying was also induced by 17β-estradiol in female slugs, implying the existence of vertebrate-type sex steroid hormones in this species (Takeda 1979, 1983). The in vitro metabolism of androstenedione and the identification of endogenous steroids (androsterone, dehydroepiandrosterone, androstenedione, 3α-androstanediol, estrone, 17β-estradiol, and estriol) by gas chromatography with mass spectrometry (GC-MS) were reported for *Helix aspersa* (Le Guellec et al. 1987). Several vertebrate-type sex steroids (androsterone, estrone, 17β-estradiol, and testosterone) and the synthetic estrogen (ethynyl estradiol) were also identified by high-resolution GC-MS in the gonads of *Thais clavigera* and *Babylonia japonica*. The detection of the synthetic estrogen, ethynyl estradiol, in the gonads, presumably represents environmental rather than endogenous origins—indicating that contamination of the habitat of *B. japonica* had occurred (Lu et al. 2001). It is therefore likely that the presence of other vertebrate-type sex steroids in *T. clavigera* and *B. japonica* may have been due to environmental exposure as opposed to synthesis in vivo.

Scott (2012) reviewed the evidence for the presence, biosynthesis, and uptake of steroids in mollusks and concluded that there was no convincing evidence for biosynthesis of vertebrate steroids by mollusks. Furthermore, Scott (2012) also pointed out in his review that the "mollusk" genome does not contain the genes for key enzymes that are necessary to transform cholesterol in progressive steps into vertebrate-type steroids. To the best of our knowledge, there has been no scientific report on steroid-producing cells in mollusks. On the other hand, there is strong evidence that mollusks are able to absorb vertebrate steroids from the environment and are able to store some of them (by conjugating them to fatty acids) for weeks to months (Scott 2012). We should also remember that the three steroids that have been proposed as functional hormones in mollusks (i.e., progesterone, testosterone, and 17β-estradiol) are the same as those of humans. Since humans (and indeed all vertebrates) continuously excrete steroids not just via urine and feces, but via their body surface (and, in fish, via the gills), it is impossible to rule out contamination as the sole reason for the presence of vertebrate steroids in mollusks (even in animals kept under supposedly "clean laboratory conditions"). Essentially, the presence of vertebrate steroids in mollusks cannot be taken as reliable evidence either of endogenous biosynthesis or of an endocrine role (Scott 2012).

Meanwhile, the biotransformation of testosterone has been characterized in the mud snail (*Ilyanassa obsoleta*) (Gooding and LeBlanc 2001). However, as there has been no scientific verification on the presence of an androgen receptor (AR) in gastropods (see below), we should perhaps interpret the biological significance of the transformation of testosterone in the *I. obsoleta* exposed at a relatively high dose (1.0 μM (150,000 DPM) [^{14}C] testosterone), with caution (Gooding and LeBlanc 2001). It is also possible that such an apparent biotransformation of high doses of steroids might be a kind of metabolism for xenobiotics in mollusks.

Aromatase-like activity has been measured and reported in several gastropod species (Morcillo and Porte 1999; Santos et al. 2002); however, the measured aromatase-like activity does not necessarily confirm the existence of vertebrate-type aromatase in gastropods. To the best of our knowledge, there has been no scientific report that has elucidated the successful isolation of aromatase protein from invertebrates. Further evidence of steroid-producing cells as well as synthetic/metabolic enzymes for steroid biosynthesis also needs to be obtained to clarify the existence of vertebrate-type sex steroid hormones in gastropods.

Although an estrogen receptor (ER)-like cDNA has been isolated from *Aplysia californica* (Gastropoda: Opisthobranchia) and the protein it encodes functions as a constitutively activated transcription factor, estrogen cannot bind this protein (Thornton et al. 2003). Similarly, an ER-like protein has also been isolated from *T. clavigera* though this too is not bound by estrogen (Kajiwara et al. 2006; Iguchi et al. 2007). This *T. clavigera* protein is also a constitutively activated transcription factor (Iguchi et al. 2007). To the best of our knowledge, no scientific report has described the successful cloning of AR from the tissues of invertebrates, including gastropods. In the absence of direct evidence for ER and AR, their physiological role in mollusks remains in doubt, even if estrogens and androgens are detected in tissues. Based on a study of fully sequenced invertebrate genomes, homologues of ER and AR have yet to be found in invertebrates (Escriva et al. 1997). Actually, recent findings of nuclear receptors in mollusks (*Crassostrea gigas*, *Biomphalaria glabrata*, and *Lottia gigantea*) also revealed that no functional nuclear receptors, such as AR and ER, have been confirmed in bivalves and gastropods, although homologues of ER and the estrogen-related receptor (ERR) were identified in them (Vogeler et al. 2014; Nordberg et al. 2014; Kaur et al. 2015). Therefore, the mollusk genome does not seem to contain genes for functioning classical nuclear steroid receptors (Scott 2012; Simakov et al. 2013; Nordberg et al. 2014). Thus, it is doubtful whether gastropods have vertebrate-type steroids as sex hormones. The absence of a molluscan AR and the constitutive expression of the ER in vitro suggest alternative pathways may exist for spermatogenesis/oogenesis in mollusks (Kaur et al. 2015). Further studies are necessary to identify steroid receptors and clarify their functions in gastropods.

On the other hand, in reviewing the evidence as to whether vertebrate sex steroids (e.g., testosterone, estradiol, progesterone) have hormonal actions in mollusks, Scott (2013) has criticized almost all related papers, in terms of their experimental designs (i.e., tested compounds or mixtures that were only presumed

to behave as steroids (or modulators of steroids) on the basis of their effects in vertebrates and pointed out neither "blinding" procedures (implying the possibility of "operator bias") nor evaluation of results (i.e., no statistical analysis)).

9.3 Involvement of the Retinoid X Receptor (RXR) and Other Nuclear Receptors in the Development of Imposex in Gastropods

Regarding the mechanism by which organotins, such as TBT and TPhT, induce the development of imposex in gastropods, as described before, six hypotheses have been proposed:

1. An increase in androgen (e.g., testosterone) levels as a result of TBT-mediated inhibition of aromatase (Bettin et al. 1996)
2. An increase in testosterone levels owing to the inhibition of acyl CoA-steroid acyltransferase (Gooding et al. 2003; Sternberg and LeBlanc 2006)
3. TBT-mediated inhibition of the excretion of androgen sulfate conjugates, with a consequent increase in androgen levels (Ronis and Mason 1996)
4. TBT interference with the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia (Féral and Le Gall 1983)
5. An increase in the level of an alanine-proline-glycine-tryptophan amide (APGWamide) neuropeptide in response to TBT (Oberdörster and McClellan-Green 2000)
6. Activation of the retinoid X receptor (RXR) (Nishikawa et al. 2004)

Experimental evidence, however, is weak for five hypotheses other than the hypothesis of (6) activation of the retinoid X receptor (RXR) (Nishikawa et al. 2004). Although it is doubtful whether gastropods have vertebrate-type steroids as sex hormones, as mentioned above in this chapter, there is also a lack of correlation between the time course of the increase in testosterone titers and penis growth in females in the aromatase inhibition hypothesis (Bettin et al. 1996; Spooner et al. 1991). Regarding the hypotheses (1), (2), and (3), Spooner et al. (1991) reported that testosterone levels were significantly elevated in TBT-exposed dogwhelks (*Nucella lapillus*) on days 28 and 42 when compared to the control, although the penis length of female *Nucella lapillus* started to increase on day 14. In another study, a combination of the aromatase inhibitor fadrozole (5 μg/g wet wt) and testosterone (0.1 μg/g wet wt) had little effect on the induction and/or promotion of imposex in *T. clavigera*, as indicated by the incidence of imposex and penis growth (Iguchi et al. 2007). Consequently, there seems uncertain about the mechanism by which organotins induce imposex in gastropods, assuming that vertebrate-type steroid hormones are involved. Meanwhile, there is a possibility that the results given in support of the "inhibition of testosterone excretion" hypothesis (Ronis and Mason 1996) may reflect a phenomenon that is at least partly short term and/or

associated with acutely toxic TBT concentrations (Matthiessen and Gibbs 1998). On the other hand, regarding the hypothesis of (2), an increase in testosterone levels owing to the inhibition of acyl CoA-steroid acyltransferase (Gooding et al. 2003; Sternberg and LeBlanc 2006), we should interpret the biological significance of the transformation of testosterone in gastropods, with caution, as mentioned above. It is also possible that such an apparent biotransformation of high doses of steroids might be a kind of metabolism for xenobiotics in mollusks (Scott 2012).

It is unknown whether aromatase-like activity is actually inhibited by TBT concentrations in tissues of gastropods collected at natural sites slightly contaminated by TBT. There is also contradictory evidence of the relationship between reduced aromatase-like activity and advance imposex symptoms in the gastropod *Bolinus brandaris* (Morcillo and Porte 1999).

Santos et al. (2005) suggested the involvement of AR, besides aromatase inhibition, in the development of imposex in *N. lapillus*. If gastropods also have AR similar to vertebrates, it may be profitable to consider the possible activation of androgen receptor-mediated responses caused by TBT or TPhT in gastropods, as the enhancements of androgen-dependent transcription and cell proliferation by TBT and TPhT have been reported in human prostate cancer cells (Yamabe et al. 2000). However, gastropods may not inherently have AR (Escriva et al. 1997; Scott 2012; Simakov et al. 2013; Vogeler et al. 2014; Nordberg et al. 2014; Kaur et al. 2015).

Several neuropeptides released from the visceral ganglia, cerebral ganglia, or the prostate gland of gastropods (e.g., *A. californica* and *Lymnaea stagnalis*) act as ovulation, egg-laying, or egg-releasing hormones (Chiu et al. 1979; Ebberink et al. 1985). Féral and Le Gall (1983) suggested that TBT-induced imposex in *O. erinacea* might be related to the release of neural morphogenetic controlling factors. Their study used in vitro tissue cultures derived from a presumed penis-forming area of the immature slipper limpet, *Crepidula fornicata*, and the isolated nervous systems of male or female *O. erinacea* in the presence/absence of TBT (0.2 μg/L) (Féral and Le Gall 1983). The accumulation of TBT or TPhT in the central nervous systems of *H. gigantea* (Horiguchi et al. 2002), *N. lapillus* (Bryan et al. 1993), and *T. clavigera* (Horiguchi et al. 2012) indicates the potential for the toxic effects of TBT and TPhT on neuroendocrine systems. Oberdörster and McClellan-Green (2000) reported that APGWamide, a neuropeptide released from the cerebral ganglia of gastropods such as *L. stagnalis*, markedly induced the development of imposex in female *I. obsoleta*. The effect of APGWamide in the induction and/or promotion of the development of imposex, however, appears weak based on the experimental results of the incidences of imposex and penis growth (Oberdörster and McClellan 2000; 2002), because the incidences of imposex and penis growth were higher and much longer in gastropods exposed to TBT and/or TPhT in the laboratory, respectively (Horiguchi 2006).

Thus, at present, five hypotheses other than the hypothesis of (6) activation of the retinoid X receptor (RXR) (Nishikawa et al. 2004) regarding the induction mechanism of imposex in gastropods cannot be fully supported.

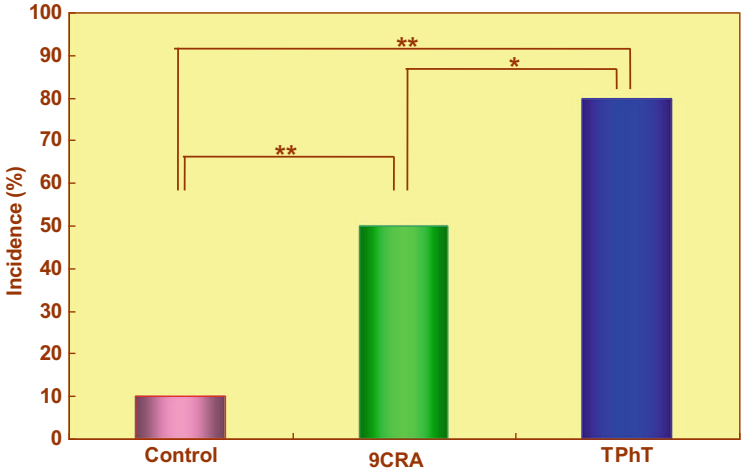

Fig. 9.1 Incidences of imposex in female rock shells (*Thais clavigera*) 1 month after treatment with fetal bovine serum (control), 9-*cis*-retinoic acid (9CRA) at 1 μg/g (wet wt.), or triphenyltin chloride (TPhT) at 1 μg/g (wet wt.). $^{*}P < 0.05$; $^{**}P < 0.01$ (Nishikawa et al. 2004)

Nishikawa et al. (2004) proposed a unique mechanism of action of TBT or TPhT on the development of imposex in gastropods, which was completely different from other hypotheses already proposed as the imposex induction mechanism. Nishikawa et al. (2004) showed that organotins (both TBT and TPhT) bound to the human retinoid X receptors (hRXRs) with high affinity and a single injection of 9-*cis* retinoic acid (9CRA), the natural ligand of hRXRs, into female rock shells (*T. clavigera*) induced the development of imposex (Figs. 9.1 and 9.2). The cloning of an RXR homologue from *T. clavigera* revealed that the ligand-binding domain of the rock shell RXR was very similar to that of the vertebrate RXR and bound to both 9CRA and organotins (Nishikawa et al. 2004). Horiguchi et al. (2008a) treated female rock shells (*Thais clavigera*) with a single injection of 3 different concentrations (0.1, 1, or 5 μg/g wet wt) of 9CRA or with a single concentration (1 μg/g wet wt) of TBT, TPhT (as positive controls), or fetal bovine serum (as a negative control) to confirm the effectiveness of 9CRA in inducing the development of imposex in *T. clavigera*. 9CRA induced imposex in a dose-dependent manner (Fig. 9.3); imposex incidence was significantly higher in the rock shells that received 1 μg ($P < 0.05$) or 5 μg ($P < 0.001$) 9CRA than in the controls. After 1 month, the rock shells treated with 5-μg 9CRA exhibited substantial growth of the penis-like structure. The length of the structure differed between the 0.1 and 5 μg 9CRA treatment groups ($P < 0.05$) but not between the 1 and 5 μg 9CRA treatment groups ($P > 0.05$). Compared with the control, the vas deferens sequence (VDS) index increased significantly in the 1 μg ($P < 0.05$) and 5 μg ($P < 0.001$) 9CRA groups. A light microscopic histological observation revealed that the penis-like structures behind the right tentacle in female rock shells treated with 5 μg 9CRA

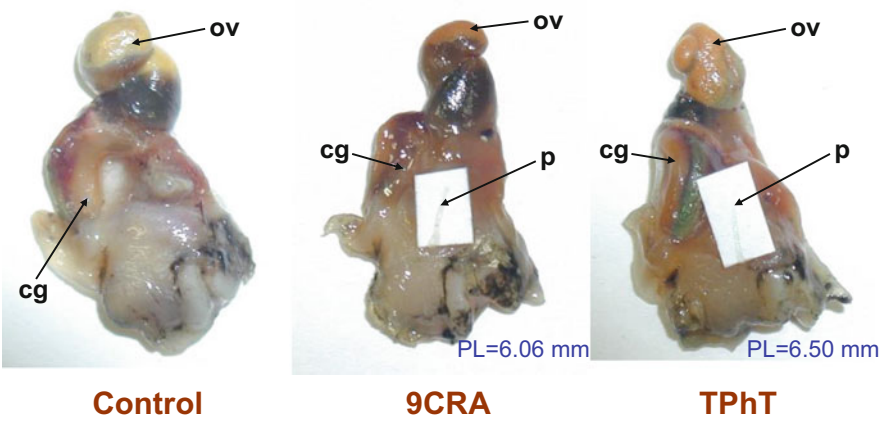

Fig. 9.2 Substantial penis growth observed in female rock shells (*Thais clavigera*) after a month of 9-*cis* retinoic acid (9CRA) injections. *cg* capsule gland, *ov* ovary, *p* penis, *PL* penis length (*Left*). Neither penis nor vas deferens was observed in the control female (after shell removal). (*Center*) Substantial penis growth as well as vas deferens development in a female that received 9CRA injection at 1 μg/g (wet wt.) (after shell removal; penis length: 6.06 mm). (*Right*) Substantial penis growth as well as vas deferens development in a positive control female that received TPhT injection at 1 μg/g (wet wt.) (after shell removal; penis length: 6.50 mm). Signs of imposex symptoms, based on penis length and the vas deferens sequence (VDS) index of females that received 9CRA injections, were clearly promoted and were similar to those in females receiving TPhT injections (Nishikawa et al. 2004)

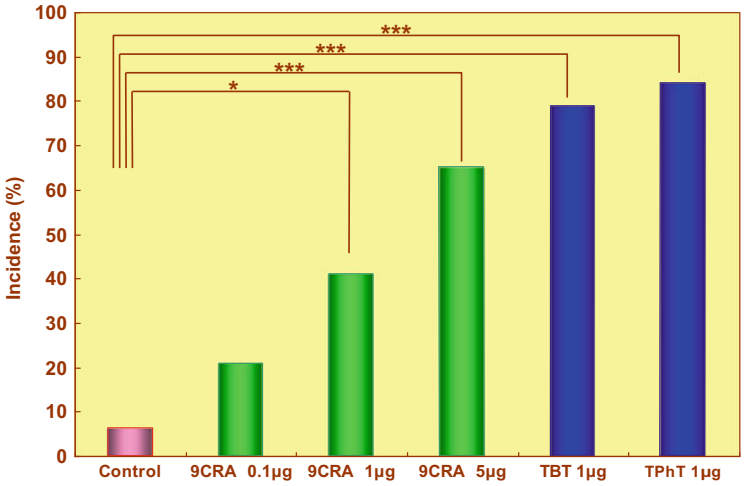

Fig. 9.3 Incidence of imposex in female rock shells (*T. clavigera*) 1 month after treatment with fetal bovine serum (Control), three different concentrations of 9-*cis*-retinoic acid (9CRA), tributyltin (TBT) chloride, or triphenyltin (TPhT) chloride. $^{*}P < 0.05$; $^{***}P < 0.001$ (Horiguchi et al. 2008a)

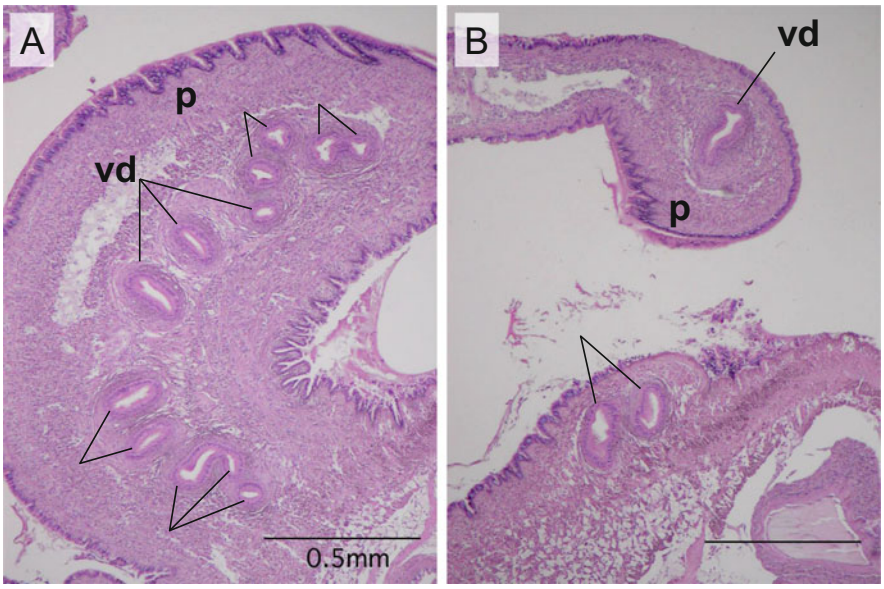

Fig. 9.4 Histology of the penis-like structure (7.00 mm long) that developed behind the right tentacle of a female rock shell (*T. clavigera*) 1 month after treatment with 9CRA at 5 μg/g (wet wt.). The sections in A and B were stained with hematoxylin and eosin. *Scale bars* represent 0.5 mm. *p* penis, *vd* vas deferens (Horiguchi et al. 2008a)

were essentially the same as the penises and vasa deferentia of normal males and of TBT-treated or TPhT-treated imposexed females (Fig. 9.4; Horiguchi et al. 2008a).

Horiguchi et al. (2007) investigated RXR gene expression and measured the RXR protein content in various tissues of wild male and female rock shells (*T. clavigera*) to further elucidate the role of RXR in the development of organotin-induced imposex in gastropod mollusks. By using the methods of quantitative real-time polymerase chain reaction, Western blotting, and immunohistochemistry with a commercial antibody against human RXR α, they revealed that RXR gene expression was significantly higher in the penises of males ($P < 0.01$) and in imposexed females ($P < 0.05$) than in the penis-forming areas of normal females (Fig. 9.5). Western blotting demonstrated that the antibody could detect rock shell RXR and showed that the male penis had the highest RXR protein content among the analyzed tissues of males and morphologically normal females. Moreover, 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 in imposexed females. Same results were also obtained, using the specific antibody for *T. clavigera* RXR (Horiguchi et al. 2010b). Based on the results of these studies, RXR could be involved in organotin-mediated induction of male-type genitalia (penis and vas deferens) in female rock shells (Horiguchi et al. 2007).

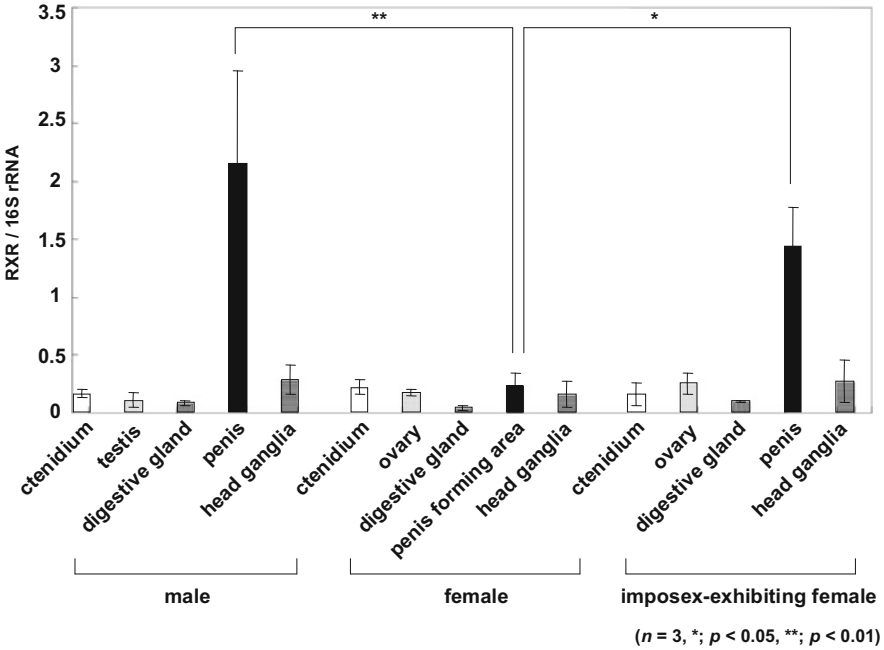

Fig. 9.5 RXR gene expression in various tissues of male, normal female, and imposex-exhibiting female rock shells (*T. clavigera*) (Horiguchi et al. 2007)

To further examine the role of RXR in the development of imposex in gastropods, Horiguchi et al. (2010a) 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 *T. clavigera* exposed to TPhT in a flow-through exposure system for 3 months. Accumulation of TPhT in the tissues was clearly observed in exposed individuals, whereas no accumulation of TPhT was observed in the control groups. In females, a 3-month exposure to TPhT 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 $\geq$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 the expression in control females; the highest RXR gene expression was found in the penis-forming area or penis (Fig. 9.6). Moreover, RXR gene expression in the penis-forming area or penis of each female exposed to TPhT seemed to be associated with penis length (Fig. 9.7). 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 penises of exposed males. These results further suggest that RXR plays an important role in

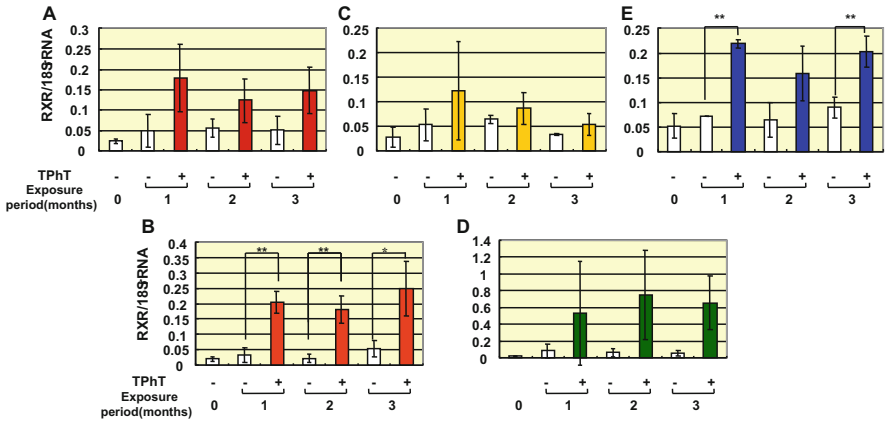

Fig. 9.6 Normalized RXR gene expression in (**A**) ctenidium, (**B**) ovary, (**C**) digestive gland, (**D**) penis-forming area or penis, and (**E**) head ganglia of female rock shells exposed to TPhT chloride at 500 ng/L for 3 months in a flow-through system. *TPhT* triphenyltin; − control females; + TPhT-exposed females; $*P < 0.05$; $**P < 0.01$ (Horiguchi et al. 2010a)

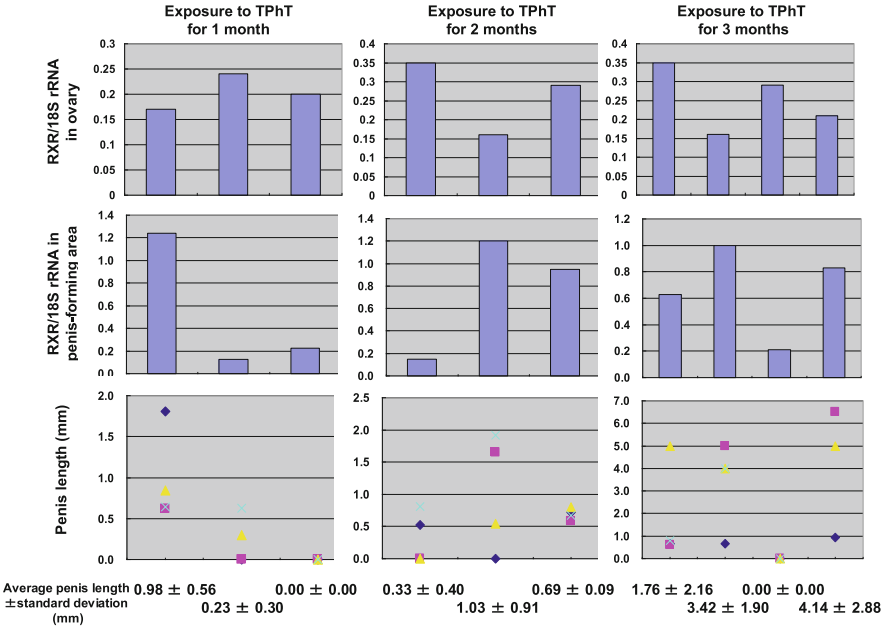

Fig. 9.7 Relationships between average penis length and RXR gene expression in penis-forming area/penis and ovary of female rock shells exposed to TPhT chloride at 500 ng/L for 3 months in a flow-through system. TPhT, triphenyltin. *Bars* in the *top* and *middle rows* represent normalized RXR gene expression in the ovary (*top row*) and penis-forming area/penis (*middle row*) of females exposed to TPhT. Dots or symbols in the *bottom row* 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 TPhT seemed to be associated with an increase in penis length. However, that in the ovary did not (Horiguchi et al. 2010a)

the development of male genitalia (i.e., penis and vas deferens) in gastropods, although RXR might also have other physiological functions.

Oehlmann et al. (2007) reviewed endocrine disruption in prosobranch gastropods, indicating and discussing its evidence and ecological relevance; they also described the results of their laboratory experiments using 9CRA. Although they injected 9CRA into female *N. lapillus*, no development of imposex was observed in specimens of *N. lapillus* examined during the experimental period (56 days), even in the highest dose (2.5 μg/g wet wt.) group. On the other hand, Castro et al. (2007) demonstrated that injections of 9CRA at 1 μg/g wet wt. in female *N. lapillus* induced the development of imposex to the same degree as did TBT (1 μg/g). In considering why contradictory results were obtained by Oehlmann et al. (2007) and Castro et al. (2007), we need to carefully examine the experimental methodologies used by each scientific group. For example, injection of 9CRA in snails should be done under shaded conditions because 9CRA is easily photodegraded.

Although Castro et al. (2007) reported that imposex in *N. lapillus* could be mediated by RXR, their experimental data showed that the level of expression of the RXR gene was highest in the gonads, unlike in the results in *T. clavigera* reported by Horiguchi et al. (2007). Castro et al. (2007) discussed the mechanism of induction of imposex by organotins in gastropods on the basis of a scenario that integrated the interaction between three cascades (retinoic, neuroendocrine, and steroid), although the physiological role of AR as well as ER in mollusks remains in doubt because no scientific report has described the successful cloning of an AR and a functional ER from the tissues of gastropods.

A recent study has provided further evidence of RXR involvement, through the cloning of RXR in the mud snail *I. obsoleta* (Sternberg et al. 2008). In light of this cloning, Sternberg et al. (2010) reviewed the mechanism of induction of imposex in prosobranch gastropods and suggested that there was environmental–endocrine control of reproductive maturation in gastropods. However, Sternberg et al. (2008) did not measure RXR gene expression in the presumptive penis-forming area or penis in females; instead, they measured it in the "the gonad-viscera complex"—probably a combination of gonadal and digestive gland tissues—although the development of imposex essentially or primarily involves the differentiation and growth of male genitalia (e.g., the penis and vas deferens). Sternberg et al. (2008) also discussed synchronized expression of RXR mRNA with recrudescence of the reproductive tract (primarily the gonad, but their samples might also have included the digestive gland) in *I. obsoleta*. Thus, their discussion seems to have confused the development of imposex (principally the differentiation and growth of the penis and vas deferens) with events of the male reproductive cycle, such as gonadal maturation and regression.

Urushitani et al. (2011) reported two isoforms of RXR cDNAs, RXR isoform 1 (*TcRXR-1*) and RXR isoform 2 (*TcRXR-2*), in the rock shell *Thais clavigera*. The deduced amino acid sequences of *TcRXR-1* and *TcRXR-2* are highly homologous with those of other gastropods. These TcRXR isoforms displayed 9CRA-dependent activation of transcription in a reporter gene assay using COS-1 cells. The transcriptional activity of TcRXR-2, the encoded protein of which has

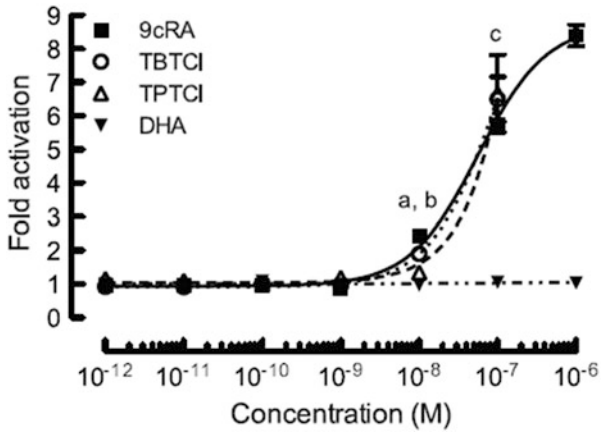

Fig. 9.8 Dose–response profiles of the activities of TcRXR-1 following exposure to various chemicals. COS-1 cells were incubated for 40–42 h with or without increasing concentrations of various ligands. *9cRA* 9-*cis* retinoic acid, *DHA* *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic acid, *TBTCl* tributyltin chloride, *TPTCl* triphenyltin chloride. Each point represents the mean of triplicate experiments. *Vertical bars* present ± S.D. [a]Significant difference between 9cRA and vehicle-treated control, [b]significant difference between TBTCl and vehicle-treated control, [c]significant difference between TPTCl and vehicle-treated control; $P < 0.01$ by ANOVA with Dunnett's post hoc test (Urushitani et al. 2011)

five additional amino acids in the T-box of the C domain, was significantly lower than that of TcRXR-1. The EC_{50} values were 1.1×10^{-7} M (95 % confidence intervals: 7.7×10^{-8} M to 1.6×10^{-7} M) in human RXR α, 6.4×10^{-8} M (5.3 to 7.9×10^{-8} M) in TcRXR-1, and 1.2×10^{-7} M (6.5×10^{-8} M to 2.2×10^{-7} M) in TcRXR-2. The induction of transcriptional activity of TcRXR-1 by 9CRA, docosahexaenoic acid (DHA), and the chemicals TBTCl and TPhTCl was analyzed, and the activity was induced by 10^{-8} M 9CRA, 10^{-8} M TBTCl, and 10^{-7} M TPhTCl but was unchanged by DHA (Fig. 9.8) (Urushitani et al. 2011). Induction of transcriptional activity of TcRXR-1 is caused by 9CRA, TBTCl, and TPhTCl, but not by DHA. It has been reported that 9CRA is a specific ligand of human RXR; however, 9CRA has not been detected in mammalian tissues (Ulven et al. 2001; Werner and DeLuca 2001), and in fiddler crabs and mollusks, its concentration is lower than those of other retinols (Dmetrichuk et al. 2008; Hopkins et al. 2008). DHA is a natural ligand in mammals, although it activates RXRs at much higher concentrations than 9CRA (de Urquiza et al. 2000). Urushitani et al. (2011) used a concentration of DHA (10^{-6} M) that was lower than that required for the induction of transcriptional activity of RXR in the mollusk *Biomphalaria glabrata* (Bouton et al. 2005), but they could not measure the transcriptional activity of TcRXR-1 induced by 10^{-4} M DHA because of toxicity. Similar results have been reported using the daphnid RXR, where DHA was also shown not to be a ligand in a reporter assay (Wang and LeBlanc 2009).

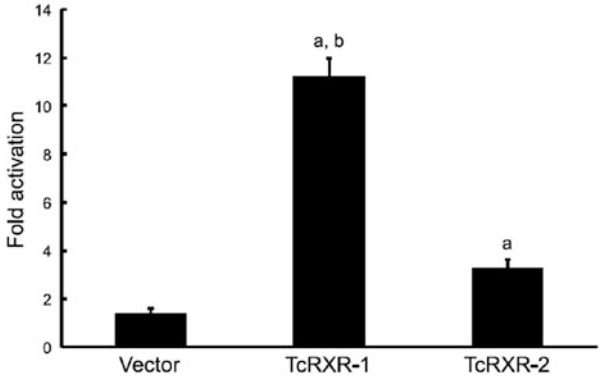

Fig. 9.9 Analysis of transcriptional activity of TcRXR isoforms. COS-1 cells were seeded into 12-well plates and then transiently transfected with the retinoid X responsible element (RXRE)-reporter vector and the *TcRXR* isoforms–fused expression vectors. COS-1 cells were incubated with or without 10^{-6} M of 9-*cis* retinoic acid (9cRA) for 40–42 h. Transcriptional activities of empty expression vector (Vector), expression vector containing *TcRXR-1* (TcRXR-1), and expression vector containing *TcRXR-2* (TcRXR-2). Data represent means ± S.D. ($n = 3$). [a]Significantly different from control, [b]significantly different from TcRXR-2; both $P < 0.01$ by Student's *t*-test (Urushitani et al. 2011)

Decreases of the transcriptional activity by TcRXR-1 were observed when more than equal amount of *TcRXR-2* fused expression vector was existed in a co-transfection assay. Overexpression of TcRXR-2 led to lower transcriptional activity than did overexpression of TcRXR-1 (Fig. 9.9). The *TcRXR* isoforms were co-transfected to investigate the effect of TcRXR-2 on the transcriptional activity of TcRXR-1 (Fig. 9.10). In these assays, significant decreases in the transcriptional activity of TcRXR-1 were observed in the presence of 0.1 or 0.5 μg *TcRXR-2*-fused expression vector (Fig. 9.10). It may imply that this difference has a functional basis in the regulation of the molluscan endocrine system (Urushitani et al. 2011). Urushitani et al. (2011) found that co-transfection of more than equal amounts of *TcRXR-2*-fused expression vector resulted in a decrease in the transcriptional activity of TcRXR-1. In mature males of *N. lapillus*, *T. clavigera*, and *I. obsoleta*, penis length varies seasonally at locations in the field that are lightly contaminated by organotins (Galante-Oliveira et al. 2010; Horiguchi et al. 2008b; Oberdörster et al. 2005). Expression of RXR mRNAs also changes seasonally in males of *T. clavigera* from lightly contaminated sites, although expression of *TcRXR-1* and *TcRXR-2* was not measured separately (Horiguchi et al. 2008b). In *I. obsoleta*, seasonal changes in RXR mRNA levels have also been reported (Sternberg et al. 2008). It was also previously reported that male penis length in *T. clavigera* decreased in the laboratory after the spawning season (Horiguchi et al. 2010a). From these findings, the interaction between TcRXR-1 and TcRXR-2 could contribute to the seasonal change in penis length in males of *T. clavigera*. Specifically, it appears that differences in the expression of *TcRXR-2* contribute to seasonal differences in penis length of male rock shells

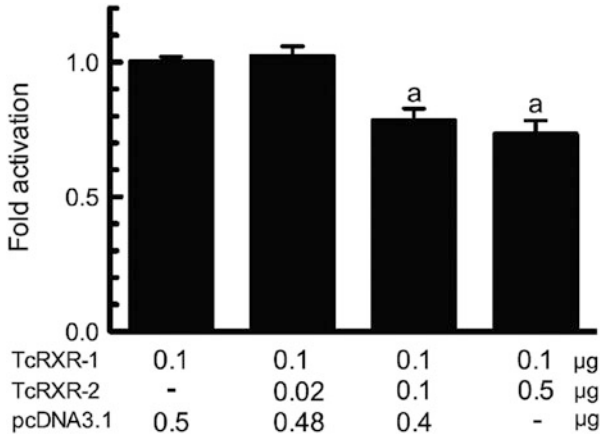

Fig. 9.10 Alterations of TcRXR isoforms' transcriptional activities. Decreases of the transcriptional activity by TcRXR-1 were observed when the amount of *TcRXR-2* was increased by more than 0.1 μg. The fold transactivation was compared with the activity of each vehicle-treated control and then normalized by the mean of the transcriptional activity for *TcRXR-1* (0.1 μg) and the pcDNA3.1 expression vector (0.5 μg). Results of triplicate luciferase assays are shown as means ± S.D. Statistically significant differences compared with the transcriptional activity of *TcRXR-1* (0.1 μg) and pcDNA3.1 expression vector (0.5 μg) in transfected cells were determined by ANOVA with Dunnett's post hoc test ([a], $P < 0.05$) (Urushitani et al. 2011)

together with possible changes in endogenous retinoids or natural ligands of TcRXRs.

These findings suggest that retinoic acids could play an important role in the development of male genitalia and their components and that RXR isoforms might underlie a novel mechanism regulating genes in gastropods. Actually, orthologs of enzymes for retinoic acid synthesis, as well as RAR sequences, have been reported in the mollusk *Lottia gigantea* (Albalat and Cañestro 2009). In vertebrates, retinoic acids and derivatives are involved in cell proliferation and differentiation, organ homeostasis, and regeneration of tissues and organs (see reviews: Albalat 2009; Chambon 1996; De Luca 1991; Kastner and Chan 2001; Kastner et al. 1995; Mark et al. 2009). All-*trans*- and 9-*cis*-RA have been detected in the mollusk *L. stagnalis* (Dmetrichuk et al. 2008). Urushitani et al. (2013) isolated a retinoic acid receptor (RAR)-like cDNA (*TcRAR*) in the rock shell, *T. clavigera*, as a candidate partner for RXR, and examined the transcriptional activity of the TcRAR protein by using all-*trans* retinoic acid (ATRA). However, no ligand-dependent transactivation by this protein was observed. Urushitani et al. (2013) also examined the transcriptional activity of the TcRAR-ligand-binding domain fused with the GAL4-DNA-binding domain by using retinoic acids, retinol, and organotins and again saw no noteworthy transcriptional induction by these chemicals. The use of a mammalian two-hybrid assay to assess the interaction of the TcRAR protein with the TcRXR isoforms suggested that TcRAR might form a heterodimer with the RXR isoforms.

The transcriptional activity of domain-swapped TcRAR chimeric proteins (the A/B domain of TcRAR combined with the D–F domain of human RARα) was also examined and found to be ATRA dependent (Urushitani et al. 2013). These results suggest that TcRAR is not activated by retinoic acids but can form a heterodimer with TcRXR isoforms.

Although 9CRA is a natural ligand for RXRs in vertebrates (Heyman et al. 1992; Mangelsdorf and Evans 1995; Mangelsdorf et al. 1992; Levin et al. 1992), whether the same is true for RXRs in *T. clavigera* or other gastropods is not clear because 9CRA is difficult to detect in vivo (Horton and Maden 1995). The natural ligand for gastropod RXR may be some compound other than 9CRA. Identification of the natural ligand for gastropod RXR is required for further analysis of the mechanism of imposex induction by organotins. Dmetrichuk et al. (2008) recently detected ATRA and 9CRA in the central nervous systems of adults of the pulmonate gastropod *L. 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 for synthesizing or transforming retinoic acids (RAs). Whether gastropods can synthesize 9CRA, ATRA, or both from β carotene must also be determined, in terms of the enzymes involved in the synthesis and metabolism of RAs (e.g., Raldh2, Cyp26). Because DHA also acts as a ligand for RXR in the brain of the fetal mouse (Urquiza et al. 2000), the possibility that DHA is a natural ligand for gastropod RXR should be examined, despite of observations of no transcriptional activity of TcRXR-1 induced by DHA (Urushitani et al. 2011).

In addition to identifying the natural ligand for gastropod RXR, we must also examine the binding and activation properties of the ligand with respect to RXR and determine whether RXR forms homodimers, homotetramers, or heterodimers with other nuclear receptors. Activation of RXR–peroxisome proliferator-activated receptor (PPAR) heterodimers by organotin compounds promotes adipocyte differentiation (Grün and Blumberg 2006; Grün et al. 2006; Kanayama et al. 2005), and the binding and activation properties of various organotins with RXR–PPAR heterodimers have been analyzed by le Maire et al. (2009). Although gastropod imposex can be induced by very low concentrations (~1 ng/L) of TBT and/or TPhT (Bryan et al. 1988; Gibbs et al. 1987; Horiguchi et al. 1994, 1995, 1997), the mechanism of how such nanomolar levels of TBT and/or TPhT can activate gastropod RXR remains to be clarified. Meanwhile, Pascoal et al. (2013) suggested additional involvement of putative PPAR pathways. Application of rosiglitazone, a well-known vertebrate PPAR γ ligand, to dogwhelks (*N. lapillus*) induced imposex in the absence of TBT. Therefore, Pascoal et al. (2013) has pointed out that it is likely also to be driven by PPAR-responsive pathways, while TBT-induced imposex is linked to the induction of many genes and has a complex phenotype.

Overall, anyway, these findings suggest that RXR is involved in the induction of male-type genitalia (penis and vas deferens) in normal male and organotin-exposed female gastropods.

We also should note that there are several steps in the development of imposex induced by certain organotin compounds, such as TBT and TPhT, in gastropods. At the initial stage of imposex development, differentiation and growth of male-type genitalia (i.e., penis and vas deferens) occur. This leads to ovarian spermatogenesis at the severely affected stage, involving oviduct blockage due to the proliferation of epidermal tissues surrounding the vas deferens (Gibbs and Bryan 1986; Gibbs et al. 1988, 1990, 1991; Horiguchi 2000; Horiguchi and Shimizu 1992; Horiguchi et al. 1994, 2000, 2002, 2005, 2006; Oehlmann et al. 1996; Schulte-Oehlmann et al. 1997). The author considers that the true mechanism of action of TBT or TPhT in the development of imposex in gastropods must encompass an explanation of each of the characteristics mentioned above (Horiguchi 2000).

It appears that the physiological regulatory system of reproduction may differ between gastropods and vertebrates. Further studies involving histological, immunohistochemical, biochemical, and molecular biological techniques are needed to elucidate the basic endocrinology and complete mechanism of action of TBT or TPhT in the development of imposex in gastropods. This may involve the identification of a natural ligand and target gene(s) of the gastropod RXR as well as the determination of when and how the differentiation and proliferation of stem cells of the penis and vas deferens in female snails are initiated and promoted, thus leading to epidermal differentiation and proliferation and the development of these organs. Morphogenetic factors could be involved in the formation of the curved penis and vas deferens. It is also possible that other factors, such as certain neuropeptides induced in the head ganglia by exposure to organotins, are associated with the RXR gene-mediated development of imposex if these factors are induced downstream of the RXR cascade (Morishita et al. 2006).

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