Section 2

Organotin Compounds as Endocrine Disruptors

Chapter 7
Mechanism of Imposex Induced by Organotins in Gastropods

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Abbreviations APGWamide: Alanine-proline-glycine-tryptophan amide; AR: Androgen receptor; ER: Estrogen receptor; GC-MS: Gas chromatography with mass spectrometry; hRXRs: Human retinoid X receptors; ILO: International Labour Organization; IPCS: International Programme on Chemical Safety; 9CRA: 9-*cis* retinoic acid; TBT: Tributyltin; TPT: Triphenyltin; UNEP: United Nations Environment Programme; VDS: Vas deferens sequence; WHO: World Health Organization

7.1 Introduction

Certain environmental chemicals cause feminization of males and/or masculinization of females, and such phenomena are generally called endocrine disruption (Colborn et al. 1996). The current status of studies of endocrine disruption both in wildlife and humans is reviewed by the International Programme on Chemical Safety (IPCS) under the joint work of the United Nations Environment Programme (UNEP), the International Labour Organization (ILO) and the World Health Organization (WHO) (International Programme on Chemical Safety 2002). Here, the author will review the masculinization of female gastropod mollusks, called imposex, in terms of the basic biology and induction mechanism of imposex.

The first report of masculinized female gastropods was made by Blaber (1970), describing a penis-like outgrowth behind the right tentacle in spent females of the dog-whelk, *Nucella lapillus* around Plymouth, UK. The term *imposex*, however, was coined by Smith (1971) to describe the syndrome of a superimposition of male type genital organs, such as the penis and vas deferens, on female gastropods. Imposex is thought to be irreversible (Bryan et al. 1986). Reproductive failure may occur in females with severe imposex, resulting in population decline or even

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mass extinction (Gibbs and Bryan 1986, 1996). In some species, imposex is typically induced by tributyltin (TBT) and triphenyltin (TPT), chemicals released from antifouling paints used on ships and fishing nets (Bryan et al. 1987, 1988; Gibbs et al. 1987; Horiguchi et al. 1995, 1997a).

As of 2004, approximately 150 gastropod species have been reported to be affected by imposex worldwide (Bech 2002a, b; Fioroni et al. 1991; Horiguchi et al. 1997b; Marshall and Rajkumar 2003; Sole et al. 1998; ten Hallers-Tjabbes et al. 2003; Terlizzi et al. 2004); many of these gastropod species belong to the families Muricidae (e.g., *N. lapillus*, *Ocenebra erinacea*, *Thais clavigera,* and *Urosalpinx cinerea*), Buccinidae (e.g., *Babylonia japonica*, *Buccinum undatum,* and *Neptunea arthritica arthritica*), Conidae (e.g., *Conus marmoreus bandanus* and *Virroconus ebraeus*), and Nassariidae (e.g., *Ilyanassa obsoleta* and *Nassarius reticulatus*) of the Neogastropoda (Fioroni et al. 1991; Horiguchi et al. 1997b).

Regarding Japanese gastropods, at least 39 species (seven mesogastropods and 32 neogastropods) have been found to be affected by imposex among 69 species examined (Horiguchi et al. 1997b; Horiguchi 2000). Although imposex has been observed mostly in shallow-water species in previous surveys, detailed studies of species living at depths of 200 m or more should also be considered, because of the discovery of imposex in Alabaster False Tun (*Galeoocorys leucodoma*) trawled from the depths of 200–250 m off the Atsumi Peninsula, Japan in 1999 (Horiguchi 2000).

The endocrine-disrupting effect of organotins on aquatic organisms is described in Chapter 8, in terms of anatomical, histopathological and ecological aspects (individual- and population-level effects).

7.2 Mode of Action of Organotin Compounds on Development of Imposex in Gastropods

7.2.1 Endocrinology of Gastropod Mollusks

Because of the lack of information on the basic biology of mollusks, understanding of reproductive physiology and/or endocrinology of gastropods is very limited. Knowledge has been mainly obtained from research on Opisthobranchia (e.g., *Aplysia californica*) and Pulmonata (e.g., *Lymnaea stagnalis*); in these gastropods several neuropeptides released from the visceral ganglia, cerebral ganglia, or the prostate gland act as hormones to promote ovulation, egg-laying, or egg-release (Chiu et al. 1979; Ebberink et al. 1985; Joosse and Geraerts 1983). There is very little understanding of the reproductive physiology and/or endocrinology of Prosobranchia (including Archaeo-, Meso- and Neogastropoda). A review by LeBlanc et al. (1999) has suggested that gastropods have both peptide and steroid hormones, however it remains unclear exactly what type of sex hormone gastropods have (see below).

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, 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 (ethynylestradiol) were also identified by high resolution GC-MS in the gonads of *T. clavigera* and *B. japonica*. The detection of the synthetic estrogen, ethynylestradiol, 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*.

In contrast, the biotransformation of testosterone has been characterized in the mud snail (*I. obsoleta*) (Gooding and LeBlanc 2001). However, as there has been no scientific verification on the presence of 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).

Further evidence of steroid-producing cells and synthetic/metabolic enzymes for steroid biosynthesis needs to be obtained to clarify the existence of vertebrate-type sex steroid hormones in gastropods. 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.

Although an estrogen receptor (ER)-like cDNA has been isolated from *A. 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 an androgen receptor (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). Thus, it remains unclear whether gastropods have AR and ER. Further studies are necessary to identify steroid receptors and clarify their functions in gastropods.

7.2.2 Mode of Action of Organotin Compounds on the Development of Imposex

Regarding the induction mechanism of imposex, several hypotheses have been proposed and they can be summarized as follows: (1) increased androgen levels, such as testosterone, due to aromatase inhibition by TBT (Bettin et al. 1996; Santos et al. 2005; Spooner et al. 1991); (2) inhibition of the excretion of sulfate conjugates of androgens by TBT (Ronis and Mason 1996); (3) disturbance of the release of penis morphogenetic/retrogressive factor from pedal/cerebropleural ganglia by TBT (Féral and Le Gall 1983); and (4) increase in a neuropeptide, alanine-proline-glycine-tryptophan amide (APGWamide) level caused by TBT (Oberdörster and McClellan-Green 2000, 2002).

Experimental evidence, however, is weak for these four hypotheses. There is a lack of correlation between the time course of the increase in testosterone titres and penis growth in females in the aromatase inhibition hypothesis (Bettin et al. 1996; Spooner et al. 1991). Regarding the hypotheses (1) and (2), Spooner et al. (1991) reported that testosterone levels were significantly elevated in TBT-exposed dog-whelks (*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.

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*, although gastropods may not inherently have AR (Escriva et al. 1997). 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 TPT in gastropods, as the enhancements of androgen-dependent transcription and cell proliferation by TBT and TPT have been reported in human prostate cancer cells (Yamabe et al. 2000).

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).

Several neuropeptides released from the visceral ganglia, cerebral ganglia, or the prostate gland of gastropods (e.g., *A. californica* and *L. 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 TPT in the central nervous systems of *H. gigantea* (Horiguchi et al. 2002), *N. lapillus* (Bryan et al. 1993), and *T. clavigera* (Horiguchi et al. 2003) indicates the potential for the toxic effects of TBT and TPT 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-Green 2000, 2002), because the incidences of imposex and penis growth were higher and much longer in gastropods exposed to TBT and/or TPT in the laboratory, respectively (Horiguchi 2006).

Thus, at present, four hypotheses regarding the induction mechanism of imposex in gastropods cannot be fully supported.

There are several characteristics in the development of imposex induced by organotin compounds, such as TBT and TPT in gastropods. At the initial stage of imposex development, the differentiation and growth of male type genital organs (i.e., penis and vas deferens) occur and lead 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). Therefore, the author considers that the true mechanism of action of TBT or TPT in the development of imposex in gastropods must encompass an explanation of each of the characteristics mentioned above (Horiguchi 2000).

Nishikawa et al. (2004) proposed a unique mechanism of action of TBT or TPT 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 TPT) bound to the human retinoid X receptors (hRXRs) with high affinity and the injection of 9-*cis* retinoic acid (9CRA), the natural ligand of hRXRs, into female rock shells (*T. clavigera*) induced the development of imposex (Figs. 7.1 and 7.2). 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. (2007b) treated female rock shells (*Thais clavigera*) with three 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, TPT (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*.

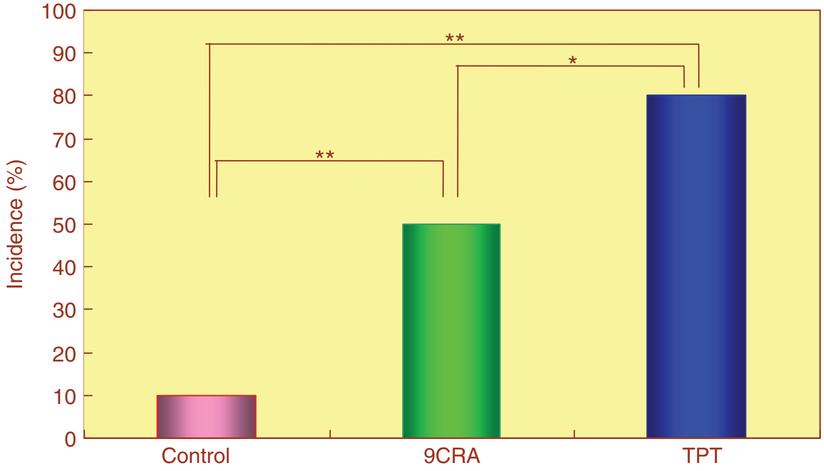

Fig. 7.1 Incidence of imposex in female rock shells (*Thais clavigera*) 1 month after treatment with fetal bovine serum (control), 1 µg/g (wet wt) of 9-*cis*-retinoc acid (9CRA), or 1 µg/g (wet wt) of triphenyltin chloride (TPT)

$^{*}P < 0.05$; $^{**}P < 0.01$

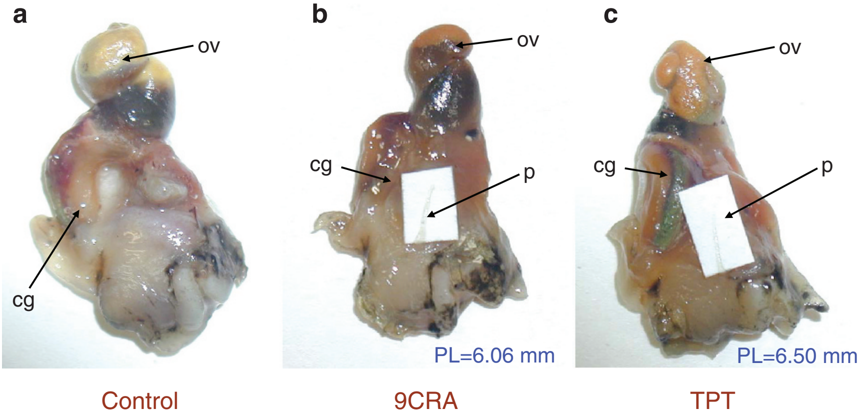

Fig. 7.2 Substantial penis growth observed in the female rock shells after a month of 9CRA injections. cg: capsule gland, ov: ovary, p: penis. (a) Neither penis nor vas deferens observed in the control female (after shell removal). (b) Substantial penis growth as well as vas deferens development observed in the female which received 1 µg/g (wet wt) of 9CRA injection (after shell removal; penis length: 6.06 mm). (c) Substantial penis growth as well as vas deferens development observed in the positive control female which received 1 µg/g (wet wt) of TPT injection (after shell removal; penis length: 6.50 mm). Imposex symptoms based on penis length and vas deferens sequence (VDS) index of the females which received 9CRA injections were clearly promoted, similar to those of females receiving TPT injections

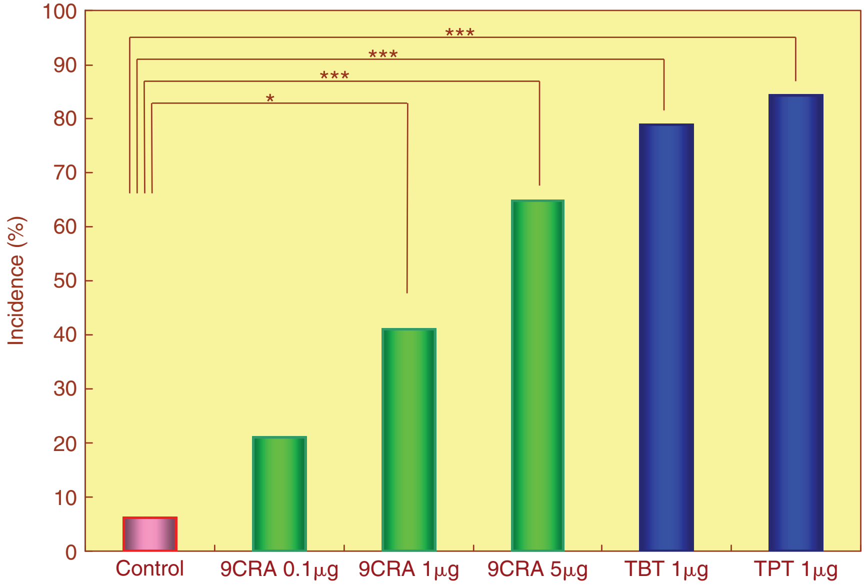

Fig. 7.3 Incidence of imposex in female rock shells (*T. clavigera*) 1 month after treatment with fetal bovine serum (control), three different concentrations of 9-*cis*-retinoc acid (9CRA), tributyltin chloride (TBT), or triphenyltin chloride (TPT)
$^{*}P < 0.05$; $^{***}P < 0.001$

9CRA induced imposex in a dose-dependent manner (Fig. 7.3); 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 μg 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 were essentially the same as the penises and vasa deferentia of normal males and of TBT-treated or TPT-treated imposexed females (Fig. 7.4; Horiguchi et al. 2007b).

Horiguchi et al. (2007a) 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 alpha (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. 7.5). Western blotting demonstrated that the antibody could detect rock shell

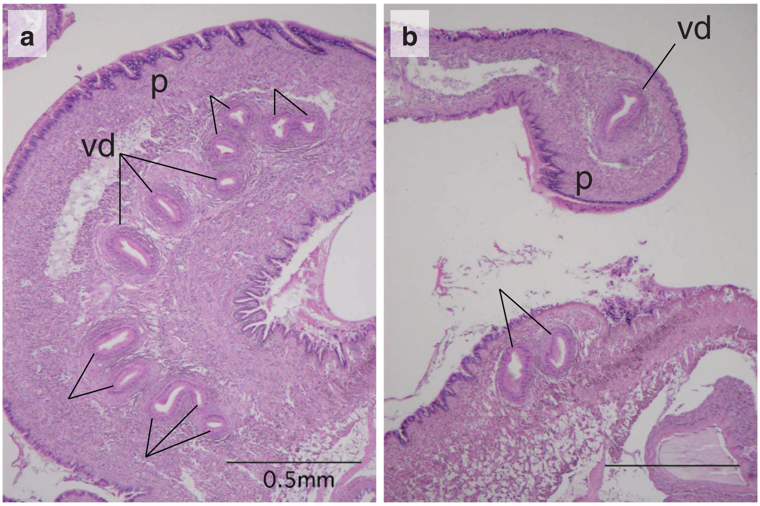

Fig. 7.4 Histology of the penis-like structure (7.00 mm in length) that developed behind the right tentacle of a female rock shell (*T. clavigera*) 1 month after treatment with 5 µg/g (wet wt) of 9CRA. The sections in a and b were stained with hematoxylin and eosin. The scale bars represent 0.5 mm. p, penis; vd, vas deferens

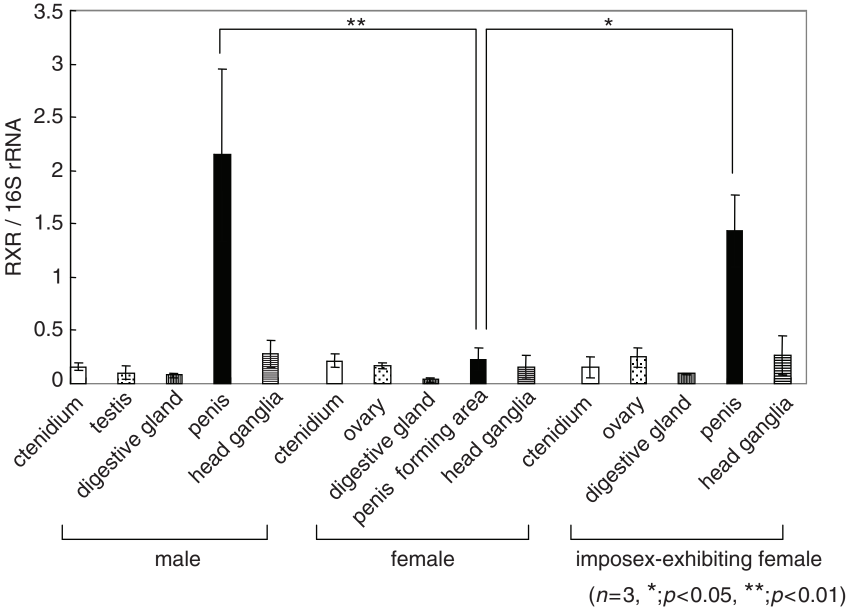

Fig. 7.5 RXR gene expression in various tissues of male, normal female, and imposex-exhibiting female rock shells (*T. clavigera*)

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 (Fig. 7.6). Based on the results of this study as well as their previous studies, Horiguchi et al. (2007a) suggested that RXR could be involved in organotin-mediated induction of male-type genitalia (penis and vas deferens) in female rock shells.

Castro et al. (2007) also reported that imposex in the dog-whelk (*N. lapillus*) could be mediated by RXR, although they observed the highest expression level of

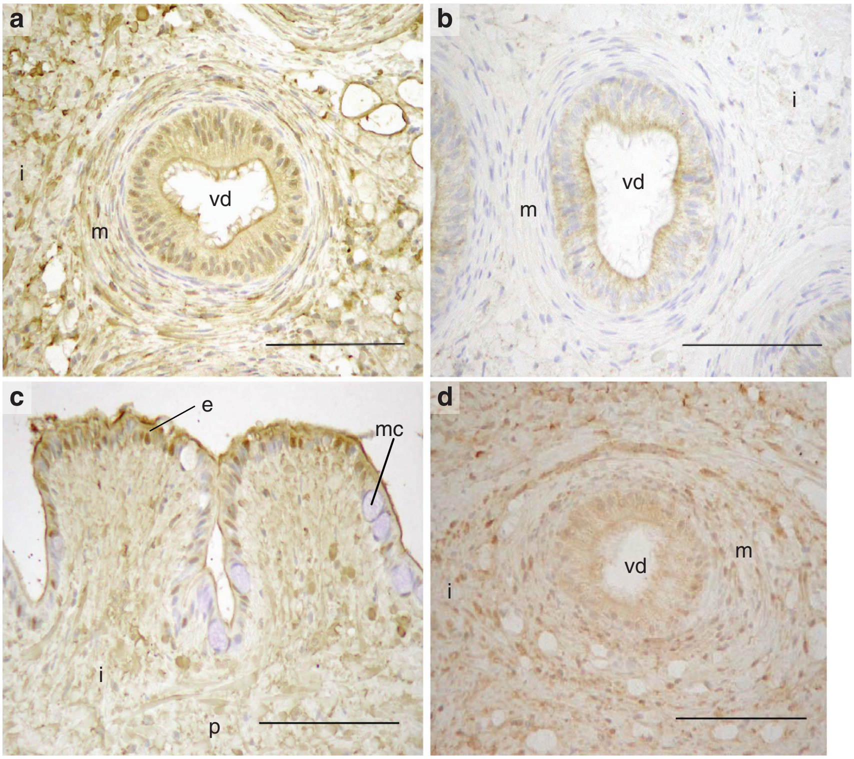

Fig. 7.6 Immunohistochemical expression of RXR in male and imposex-exhibiting female rock shells (*T. clavigera*). Males and severely imposexed females collected at Jogashima (a contaminated site) in December 2003 (a–e). Normal females collected at Hiraiso (a reference site) in December 2003 (h). Slightly affected imposex-exhibiting females collected at Hiraiso in December 2004 (f and g). (a) Penis of a male, showing positive nuclear staining in the epithelial cells of the vas deferens and the surrounding smooth muscle. (b) Penis of a male stained with antibody neutralized with blocking peptide, showing no staining. Non-specific staining was visible in the superficial region of the epithelium of the vas deferens. (c) Epidermal region of penis in a male showing positive staining of epithelial cells. Staining of the superficial layer of the epidermis was non-specific. (d) Penis of a severely affected imposex-exhibiting female, showing similar RXR expression to that in the male (a).

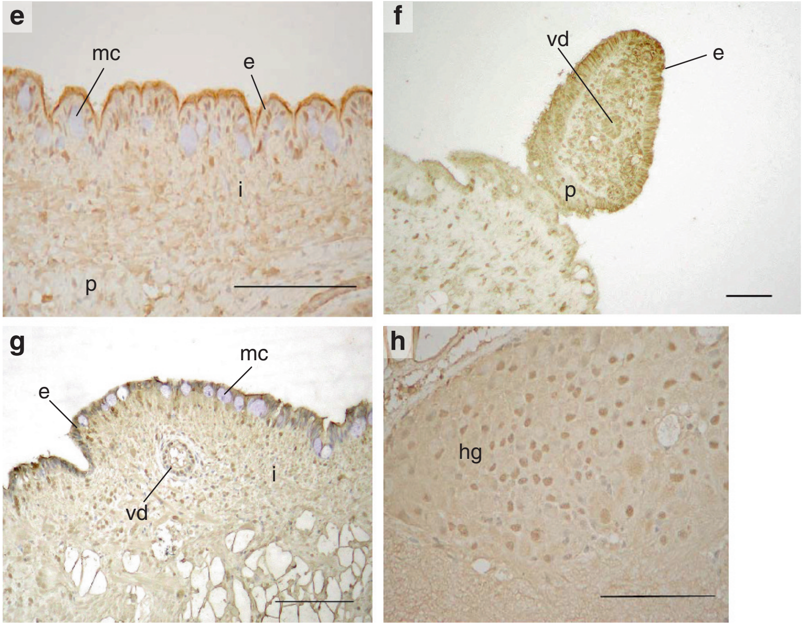

Fig. 7.6 (continued) (e) Epidermal region of penis in a severely imposexed female, showing positive staining in the epidermal cells. (f) Tiny penis of a slightly affected imposex-exhibiting female, showing positive staining in the epithelial cells of the vas deferens, and in the epidermal and interstitial cells. (g) Epidermal region behind the right tentacle of a slightly affected imposex-exhibiting female, showing positive staining in the epithelial cells of the developing vas deferens, and in the epidermal and interstitial cells. (h) Head ganglia of a normal female, showing positive staining in the nerve cells. Sections were counterstained with Hematoxylin. Scale bars indicate 100 µm. e, epithelium (or epithelial cell); hg, head ganglia; i, interstitial tissue (or interstitial cell); m, muscle layer (or smooth muscle cell); mc, mucous cell; p, penis; vd, vas deferens

RXR gene in gonads, which was different from the rock shell (*T. clavigera*). They discussed the induction mechanism of imposex caused by organotins in gastropods, on the basis of a complicated scenario that integrates the interaction between three cascades (retinoic, neuroendocrine and steroid).

Overall, however, these findings suggest that RXR plays an important role in inducing the development of imposex, namely the differentiation and growth of male type genital organs in female gastropods.

Preliminary experimental results on RXR gene expression and induction of imposex after 3-month flow-through exposure to TPT with the rock shell (*T. clavigera*) further support the hypothesis that RXR plays an important role in inducing the development of imposex caused by organotins in female gastropods (Horiguchi et al., manuscript in preparation).

Further studies involving histological, immunohistochemical, biochemical and molecular biological techniques are needed to elucidate the complete mechanism of action of TBT or TPT on the development of imposex in gastropods. This may involve the clarification of a natural ligand and target gene(s) of the rock shell RXR, and when and how the differentiation and proliferation of the stem cells of the penis and vas deferens in a female rock shell are initiated and promoted, which could lead to the epidermal differentiation and proliferation of the penis and vas deferens formation. 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, might be associated with the RXR gene-mediated development of imposex – if these factors are induced downstream of the RXR cascade (Morishita et al. 2006).

7.3 Conclusions

It appears that the physiological regulatory system of reproduction may be different in gastropods, compared to that of vertebrates. The retinoid X receptor (RXR) has an important role in the TBT and/or TPT-induced development of imposex, and the subsequent differentiation and growth of male type genital organs, in female gastropods.

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