

Establishment of a polyclonal antibody against the retinoid X receptor of the rock shell *Thais clavigera* and its application to rock shell tissues for imposex research

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Abstract In the chain of study to further elucidate the role of retinoid X receptor (RXR) in the development of imposex caused by organotin compounds in gastropod mollusks, we established a polyclonal antibody against RXR of the rock shell *Thais clavigera*. Immunoblotting demonstrated that this antibody could recognize *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 polyclonal antibody against *T. clavigera* RXR can specifically recognize RXR protein in tissues of *T. clavigera* and therefore is useful for evaluating RXR protein localization. Furthermore, RXR may be involved in the induction of male-type genitalia (penis and vas deferens) in normal male and organotin-exposed female rock shells.

Keywords Imposex · Polyclonal antibody · Retinoid X receptor (RXR) · Rock shell · *Thais clavigera*

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Introduction

Imposex is an irreversible pseudohermaphroditic condition in which male genital organs, such as the penis and vas deferens, develop in female gastropods (Bryan et al. 1986; Smith 1971). Imposex is typically induced by very low concentrations (~1 ng/L) of tributyltin (TBT), triphenyltin (TPT), or both, which have been used in antifouling paints for ships and fishing nets since the mid-1960s (Bryan et al. 1986, 1987, 1988; Gibbs et al. 1987; Horiguchi et al. 1994, 1997a). Reproductive failure occurs in the severe stages of imposex, either because of oviduct blockage by the formation of vasa deferentia or because of ovarian spermatogenesis, and eventually results in population decline and/or mass extinction (Gibbs and Bryan 1986; Gibbs et al. 1988, 1990; Horiguchi et al. 2006). Globally, more than 150 species of mesogastropods and neogastropods, including the rock shell *Thais clavigera*, are affected by imposex (Fioroni et al. 1991; Horiguchi et al. 1997b, 2006; Matthiessen et al. 1999). Imposex among gastropods has been reported to be a clear manifestation of endocrine disruption (Matthiessen and Gibbs 1998; Matthiessen et al. 1999).

Four hypotheses regarding the mechanisms by which organotins induce imposex in gastropods 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) TBT-mediated inhibition of the excretion of androgen sulfate conjugates (Ronis and Mason 1996); (3) TBT interference in the release of penis morphogenetic/retrogressive factor from the pedal/cerebropleural ganglia (Féral and Le Gall 1983); and (4) an increase in the level of alanine-proline-glycine-tryptophan amide neuropeptide in response to TBT (Oberdörster and McClellan-Green 2000). However, the scientific debate

continues on the mechanisms by which organotins induce imposex in gastropods because, as noted by Horiguchi et al. (2008) and Horiguchi (2009), each of these hypotheses is insufficient to explain the phenomenon.

Nishikawa et al. (2004) found that *T. clavigera* has a retinoid X receptor (RXR) that is similar to those in humans and other vertebrates as well as to those in other invertebrates, such as ascidians, insects, pulmonates, jellyfish, and sponges (Bouton et al. 2005; Devine et al. 2002; Freebern et al. 1999; Heyman et al. 1992; Kamimura et al. 2000; Kostrouch et al. 1998; Mangelsdorf and Evans 1995; Mangelsdorf et al. 1992; Nagatomo et al. 2003; Wiens et al. 2003). Nishikawa et al. (2004) also observed that *T. clavigera* RXR binds to both 9-*cis*-retinoic acid (9CRA), which is known to be a natural ligand for human RXRs, and organotins. In addition, they demonstrated that a single *in vivo* injection of 9CRA into female rock shells without morphological signs of imposex induced the development of imposex a month later. Horiguchi et al. (2007) investigated RXR gene expression and measured the RXR protein content in various tissues of wild male and female *T. clavigera* using quantitative real-time polymerase chain reaction (PCR), Western blotting, and immunohistochemistry with a commercial antibody against human RXR α . Based on the results of this study, Horiguchi et al. (2007) suggested that RXR could be involved in organotin-mediated induction of male-type genitalia (penis and vas deferens) in female rock shells.

In the present study, in the chain of study to further elucidate the role of RXR in the development of imposex caused by organotin compounds in gastropods, we established a polyclonal antibody against RXR of *T. clavigera* and confirmed that this antibody could detect *T. clavigera* RXR by immunoblotting. Immunohistochemical staining was applied to examine the localization of RXR protein in tissues of the rock shell, using this antibody.

Materials and methods

Production of a polyclonal antibody against RXR of the rock shell

Full-length *T. clavigera* RXR (AY704160) was synthesized as a recombinant protein with a tag of glutathione *S*-transferase (GST) in *Escherichia coli* (BL21(DE3)LysS) and was injected into two rabbits (SPF Japanese white rabbit). The antibody titer was measured by ELISA 2 months after injection to confirm that the titer was sufficiently increasing. Antibody was affinity purified from the antiserum, using GST-RXR affinity column.

Detection of RXR protein using immunoblotting

Construction of plasmid vectors

The full-coding regions of *T. clavigera* RXR and human RXR α (NM_002957) were amplified by PCR with KOD Plus DNA polymerase (Toyobo Biochemicals, Osaka, Japan). The PCR product was gel-purified and subcloned into pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA).

Transient expression and immunoblotting assay

To examine the characteristics of anti-*T. clavigera* RXR antibody, COS-1 cells were seeded in 6-well plates at 5×10^5 cells per well in phenol-red-free DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone, South Logan, UT, USA). After 24 h, the cells were transfected with 2 μ g pcDNA3.1(+) vector fused to *T. clavigera* RXR or human RXR, and empty pcDNA3.1(+) vector using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. After 40 h, the cells were washed twice with phosphate-buffered saline (8.1 m mol/L Na_2HPO_4 , 1.47 m mol/L KH_2PO_4 , 2.68 m mol/L KCl, 137 m mol/L NaCl), harvested, lysed in SDS sample buffer, and then analyzed on 10% Laemmli SDS-polyacrylamide gel (Laemmli 1970). The cell lysates were sonicated and then incubated at 70°C for 10 min. Equal volumes of cell lysates were subjected to 10% SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA, USA) by electroblotting (Towbin et al. 1979). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (TBS: 20 m mol/L Tris-HCl, 150 m mol/L NaCl, pH 7.5) and incubated with each primary antibody diluted in TBS (anti-*T. clavigera* RXR, 1 μ g/mL; anti-human RXR Δ N197 [Santa Cruz Biotechnology, Santa Cruz, CA, USA], 1:200; anti- β -actin [clone AC-15: Sigma-Aldrich], 1:1000). After washing (5 min \times 3) with TBS containing 0.1% Tween 20 (TTBS), membranes were incubated with an alkaline phosphatase-conjugated secondary antibody (anti-mouse IgG antibody, 1:1000; anti-rabbit IgG antibody, 1:1000; both from Invitrogen). Following another three washes with TTBS, phosphatase activity was visualized by treating the membrane with 0.2 mM 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt and nitro blue tetrazolium (Sigma-Aldrich) in 100 m mol/L diethanolamine buffer (pH 9.5) containing 5 m mol/L MgCl_2 . All incubations were performed at room temperature.

Immunohistochemical staining for RXR

In April 2008 both male and female wild rock shells (approximately 300 individuals) were collected at Aikawa on Sado Island in Niigata Prefecture, Japan (38° 01' 39.75'' N, 138° 14' 18.48'' E; a reference site known to have less than 11 and 8 ng/g wet wt. TBT and TPT, respectively; see Horiguchi et al. 1994). In May 2009 wild imposex-exhibiting female rock shells (approximately 30 individuals) were collected at Hinase in Okayama Prefecture, Japan (34° 43' 46.54' N, 134° 16' 06.01'' E; a site severely contaminated with TBT and TPT; e.g., tissue concentrations of TBT and TPT in rock shells were 116.4 and 174.2 ng/g wet wt., respectively; see Horiguchi 2004). After collection, the rock shells were immediately dissected and then preserved by immersion in 10% phosphate-buffered formalin at pH 7.4 for 48 h.

The tissues were dehydrated, embedded in paraffin, and sectioned to 4 μm. To enhance their antigenicity, deparaffinized and rehydrated sections were immersed in 0.8 M urea and autoclaved at 121°C for 15 min. Sections were then treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature and blocked in serum-free blocking solution (DakoCytomation, Dako, Glostrup, Denmark) for 30 min at room temperature. After incubation with anti-*T. clavigera* RXR antibody (dilution 1:1000) for 16 h at 4°C, sections were visualized with a labeled avidin–biotin system, using conjugated horseradish peroxidase (LSAB kit, Dako) and 0.01% 3,3-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan) in 50 mM Tris–HCl (pH 7.6) containing 0.068% imidazole (Sigma–Aldrich) and 0.02% hydrogen peroxide. Control sections were incubated with normal rabbit serum (Dako) instead of the anti-RXR antibody. All sections were counterstained with Mayer’s hematoxylin.

Results

Production of a polyclonal antibody against RXR of the rock shell

Two sets of approximately 90 mL of affinity-purified antiserum (89 and 86 mL for rabbits no. 1 and 2, respectively) were obtained. The antibody titer measured by ELISA is shown in Fig. 1. The values of OD450 were over 1.1 in the antisera at a dilution of 1:12,500, and the values were around 0.9 even in the antisera at a dilution of 1:62,500 (Fig. 1). Affinity-purified antiserum of rabbit no. 2 was selected to use in the following experiments.

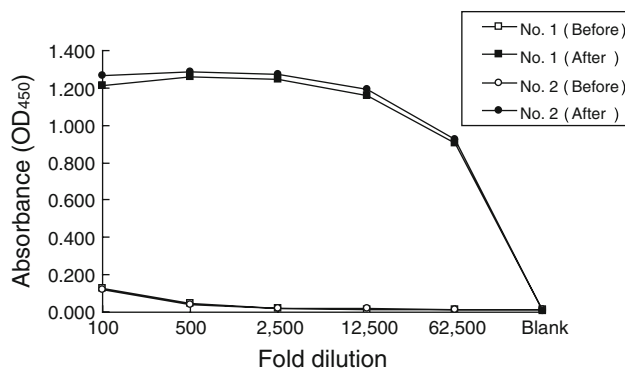


Fig. 1 The antibody titers in antisera of two rabbits against RXR of the rock shell *Thais clavigera* measured by ELISA. Antisera were diluted in 100–62,500 folds. Before, before antigen injections; After, approximately 2 months after antigen injection

Detection of RXR protein using immunoblotting

The reaction was represented as a major band of approximately 50 kDa (Fig. 2a, b). Because the molecular weight of *T. clavigera* RXR is approximately 49 kDa, immunoblotting demonstrated that this antiserum against *T. clavigera* RXR could detect *T. clavigera* RXR (Fig. 2a, b). Anti-*T. clavigera* RXR antibody strongly recognized RXR in extracts from *T. clavigera* RXR-expressed culture cells but not in those from human RXR α-expressed cells (Fig. 2a). Using anti-human RXR α antibody, which recognized a range of domains from the DNA-binding domain to the F domain of human RXRs, we found expression of both *T. clavigera* and human RXRs (Fig. 2c). Thus, this antiserum against RXR of the rock shell was able to specifically detect RXR protein in tissues of *T. clavigera*.

Immunohistochemical staining for RXR: localization of RXR in penis and vas deferens of male and imposex-exhibiting female rock shells

Immunohistochemical staining with anti-serum against *T. clavigera* RXR revealed the localization of RXR in the

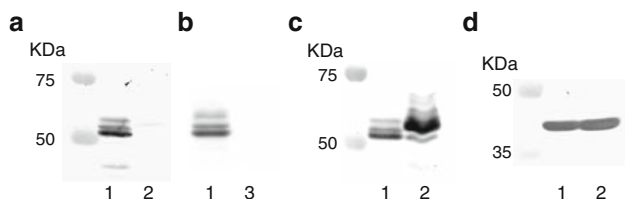


Fig. 2 Specification of anti-*Thais clavigera* RXR antibody. COS-1 cells were transiently expressing (1) *T. clavigera* RXR, (2) human RXR α and (3) pcDNA3.1(+). Equal volumes of cell lysates were analyzed by immunoblotting using (a, b) anti-*T. clavigera* RXR antibody, (c) anti-human RXR α antibody, and (d) anti-β-actin antibody

penis of wild males and imposex-exhibiting females (Fig. 3). In the vas deferens and penis in wild males, RXR was invariably detected in the nuclei of epithelial cells and in the cells of the smooth muscle layer surrounding the epithelium, as well as in cells in the interstitial tissue (Fig. 3b). Nuclear staining was also seen in the epidermis. No immunoreactivity was detected in any tissues treated with normal rabbit serum as negative controls (Fig. 3a), confirming binding specificity. In females exhibiting severe imposex, RXR expression was essentially the same as that in males, appearing in the nuclei of the epithelial cells lining the vas deferens and the surrounding smooth muscle cells (Fig. 3c). RXR was also evident in some of the nerve cells in the head ganglia of normal males (Fig. 3d), normal females, and in those of females showing imposex. Localization of RXR in penis and vas deferens of male and imposex-exhibiting female rock shells as well as localization of RXR in nerve cells in the head ganglia of rock shells were similar to findings previously reported by Horiguchi et al. (2007).

Discussion

In this study, we established a polyclonal antibody against RXR of *T. clavigera* as a specific antibody that recognizes *T. clavigera* RXR protein. This antibody could be very useful in analyses of the mode of action of organotin compounds, such as TBT and TPT, during their induction of imposex in gastropods. Horiguchi et al. (2007) reported localization of RXR in several tissues of *T. clavigera* on the basis of immunohistochemical staining with anti-human RXR α polyclonal antibody (D-20, Santa Cruz Biotechnology). The present study has confirmed the localization of RXR in penis and vas deferens of male and imposex-exhibiting female rock shells as well as in nerve cells in the head ganglia, results that are similar to those of Horiguchi et al. (2007). Thus, our hypothesis that RXR is involved in the differentiation of male-type genitalia in normal male rock shells and organotin-exposed females is further supported by these results.

RXR gene expression was observed previously in various tissues of the males, normal females, and imposex-

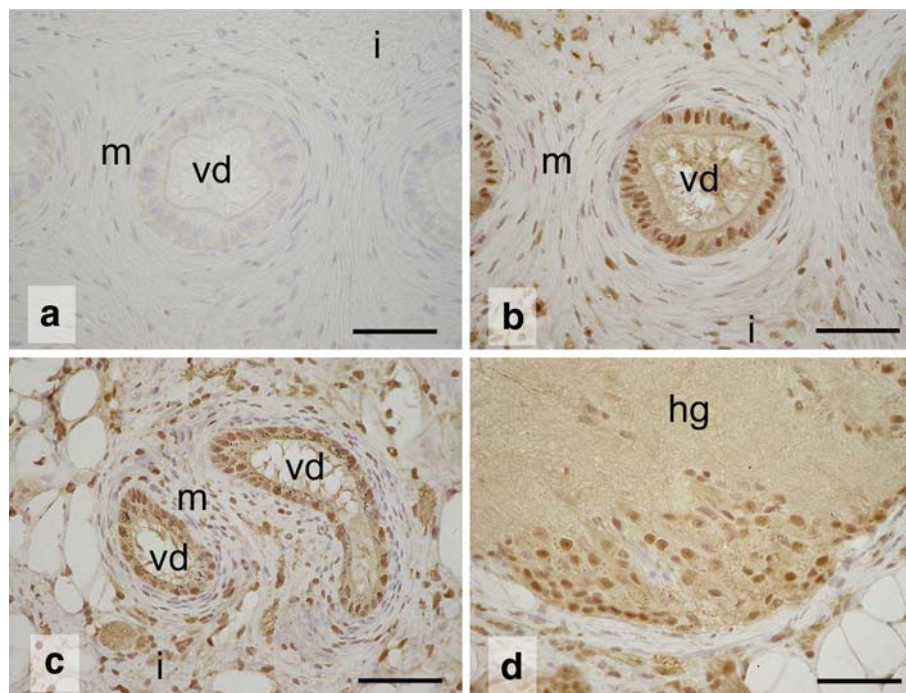


Fig. 3 Immunohistochemical expression of RXR in male and imposex-exhibiting female *T. clavigera*. Males and imposex-exhibiting females were collected at Aikawa (reference site, April 2008) and Hinase (contaminated site, May 2009), respectively. **a** Penis of a male stained with normal rabbit serum without antibody, showing no staining. **b** Penis of a male stained with anti-*T. clavigera* RXR antibody, showing positive nuclear staining in the epithelial cells of the vas deferens, smooth muscle cells surrounding the epithelium, and

interstitial cells. **c** Penis of a severely affected imposex-exhibiting female, showing similar RXR expression to that in the male of (**b**). **d** Head ganglion (pedal ganglion) of a normal male, showing positive staining in the nerve cells. Sections were counterstained with hematoxylin. Scale bars indicate 50 μ m. hg, head ganglion; i, interstitial tissue (or interstitial cell); m, muscle layer (or smooth muscle cell); vd, vas deferens

exhibiting females examined (Horiguchi et al. 2007). RXR protein was also detected in penis and vas deferens, as well as the head ganglia (Fig. 3), suggesting that the tissue distribution of RXR in *T. clavigera* is extensive. This may indicate that RXR has multiple physiological functions or roles in various tissues of the rock shell, similar to vertebrates, where retinoic acids (RAs) play key roles in embryo patterning and organogenesis (Morris-Kay 1997; Redfern 1997).

To further analyze the induction mechanism of imposex caused by organotins in gastropods, the natural ligand to *T. clavigera* RXR and the possibility that *T. clavigera* RXR forms a heterodimer by coupling with other nuclear receptors (e.g., retinoic acid receptor (RAR), thyroid hormone receptor, vitamin D receptor, and peroxisome-proliferator-activated receptor (PPAR)) needs to be identified (Horiguchi et al. 2007).

Dmetrichuk et al. (2008) recently detected all-*trans* retinoic acid (ATRA) and 9CRA in the central nervous system of adults of the pulmonate gastropod *Lymnaea stagnalis* by high-performance liquid chromatography/mass spectrometry. Because ATRA and 9CRA were detected in tissue of *L. stagnalis*, the species likely also has metabolic enzymes to synthesize or transform RAs. The presence of 9CRA, a possible candidate as a natural ligand for RXR, should be investigated in the prosobranch gastropod *T. clavigera*. It is also necessary to examine whether the rock shell can inherently synthesize 9CRA and/or ATRA from β carotene; this may not be the case in light of the fact that no report has described the genes encoding RAR or the enzymes involved in the synthesis and degradation/metabolism of RAs (e.g., Raldh2, Cyp26) in invertebrates, except in the Prochordata (e.g., ascidians and amphioxus; Escriva et al. 1997; Nagatomo and Fujiwara 2003).

The activation of RXR–PPAR heterodimers by organotin compounds is considered to promote 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 were analyzed by le Maire et al. (2009). Gastropod imposex is induced by very low concentrations (~ 1 ng/L) of TBT and/or TPT (Bryan et al. 1986, 1987, 1988; Gibbs et al. 1987; Horiguchi et al. 1994, 1997a), but the mechanism of how such nanomolar levels of TBT and/or TPT can activate *T. clavigera* RXR remains to be clarified.

We also must examine the physiological and biochemical responses downstream of the RXR signaling pathway in order to elucidate the mechanisms of RXR-mediated transcription regulation. Such knowledge would improve our understanding of the entire mechanism of induction of imposex caused by organotin compounds, such as TBT and TPT, in gastropods, including the differentiation, growth

and formation of male-type genitalia in normal male and organotin-exposed female rock shells.

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References

- Bettin C, Oehlmann J, Stroben E (1996) TBT-induced imposex in marine neogastropods is mediated by an increasing androgen level. *Helgol Meeresunters* 50:299–317
- 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) A conserved retinoid X receptor (RXR) from the mollusk *Biomphalaria glabrata* transactivates transcription in the presence of retinoids. *J Mol Endocrinol* 34:567–582
- Bryan GW, Gibbs PE, Hummerstone LG, Burt GR (1986) The decline of the gastropod *Nucella lapillus* around south-west England: evidence for the effect of tributyltin from antifouling paints. *J Mar Biol Assoc UK* 66:611–640
- Bryan GW, Gibbs PE, Burt GR, Hummerstone LG (1987) The effects of tributyltin (TBT) accumulation on adult dog-whelk, *Nucella lapillus*: long-term field and laboratory experiments. *J Mar Biol Assoc UK* 67:525–544
- Bryan GW, Gibbs PE, Burt GR (1988) A comparison of the effectiveness of tri-*n*-butyltin chloride and five other organotin compounds in promoting the development of imposex in the dog-whelk, *Nucella lapillus*. *J Mar Biol Assoc UK* 68:733–744
- Devine C, Hinman VF, Degnan BM (2002) Evolution and developmental expression of nuclear receptor genes in the ascidian *Herdmania*. *Int J Biol* 46:687–692
- Dmetrichuk JM, Carlone RL, Jones TRB, Vesprini ND, Spencer GE (2008) Detection of endogenous retinoids in the molluscan CNS and characterization of the trophic and tropic actions of 9-*cis* retinoic acid on isolated neurons. *J Neurosci* 28:13014–13024
- Escriva H, Safi R, Hanni C, Langlois MC, Saumitou-Laprade P, Stehelin D, Capron A, Pierce R, Laudet V (1997) Ligand binding was acquired during evolution of nuclear receptors. *Proc Natl Acad Sci USA* 94:6803–6808
- Féral C, Le Gall S (1983) The influence of a pollutant factor (TBT) on the neurosecretory mechanism responsible for the occurrence of a penis in the females of *Ocenebra erinacea*. In: Lever J, Boer HH (eds) *Molluscan neuro-endocrinology*. North-Holland Publishing, Amsterdam, pp 173–175
- Fioroni P, Oehlmann J, Stroben E (1991) The pseudohermaphroditism of prosobranch: morphological aspects. *Zool Anz* 226:1–26
- Freebern WJ, Osman A, Niles EG, Christen L, LoVerde PT (1999) Identification of a cDNA encoding a retinoid X receptor homologue from *Schistosoma mansoni*: evidence for a role in female-specific gene expression. *J Biol Chem* 274:4577–4585
- Gibbs PE, Bryan GW (1986) Reproductive failure in populations of the dog-whelk, *Nucella lapillus*, caused by imposex induced by tributyltin from antifouling paints. *J Mar Biol Assoc UK* 66:767–777
- Gibbs PE, Bryan GW, Pascoe PL, Burt GR (1987) The use of the dog-whelk, *Nucella lapillus*, as an indicator of tributyltin (TBT) contamination. *J Mar Biol Assoc UK* 67:507–523
- Gibbs PE, Pascoe PL, Burt GR (1988) Sex change in the female dog-whelk, *Nucella lapillus*, induced by tributyltin from antifouling paints. *J Mar Biol Assoc UK* 68:715–731

- Gibbs PE, Bryan GW, Pascoe PL, Burt GR (1990) Reproductive abnormalities in female *Ocenebra erinacea* (Gastropoda) resulting from tributyltin-induced imposex. *J Mar Biol Assoc UK* 70:639–656
- Grün F, Blumberg B (2006) Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology* 147:S50–S55
- Grün F, Watanabe H, Zamanian Z, Maeda L, Arima K, Cubacha R, Gardiner DM, Kanno J, Iguchi T, Blumberg B (2006) Endocrine-disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. *Mol Endocrinol* 20:2141–2155
- Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, Thaller C (1992) 9-*cis*-retinoic acid is a high-affinity ligand for the retinoid X receptor. *Cell* 68:397–406
- Horiguchi T (2004) Gastropods. In: Takeuchi I, Tanabe S, Hino A (eds) Biological monitoring for anthropogenic chemicals in low doses. Koseisha-Koseikaku, Tokyo, pp 37–67 (in Japanese)
- Horiguchi T (2009) Mechanism of imposex induced by organotins in gastropods. In: Arai T, Harino H, Ohji M, Langston WJ (eds) Ecotoxicology of antifouling biocides. Springer, Tokyo, pp 111–124
- Horiguchi T, Shiraishi H, Shimizu M, Morita M (1994) Imposex and organotin compounds in *Thais clavigera* and *T. bronni* in Japan. *J Mar Biol Assoc UK* 74:651–669
- Horiguchi T, Shiraishi H, Shimizu M, Morita M (1997a) Effects of triphenyltin chloride and five other organotin compounds on the development of imposex in the rock shell, *Thais clavigera*. *Environ Pollut* 95:85–91
- Horiguchi T, Shiraishi H, Shimizu M, Morita M (1997b) Imposex in sea snails, caused by organotin (tributyltin and triphenyltin) pollution in Japan: a survey. *Appl Organomet Chem* 11:451–455
- Horiguchi T, Kojima M, Hamada F, Kajikawa A, Shiraishi H, Morita M, Shimizu M (2006) Impact of tributyltin and triphenyltin on ivory shell (*Babylonia japonica*) populations. *Environ Health Perspect* 114(Suppl. 1):13–19
- Horiguchi T, Nishikawa T, Ohta Y, Shiraishi H, Morita M (2007) Retinoid X receptor gene expression and protein content in tissues of the rock shell *Thais clavigera*. *Aquat Toxicol* 84:379–388
- Horiguchi T, Ohta Y, Nishikawa T, Shiraishi F, Shiraishi H, Morita M (2008) Exposure to 9-*cis* retinoic acid induces penis and vas deferens development in the female rock shell, *Thais clavigera*. *Cell Biol Toxicol* 24:553–562
- Kamimura M, Fujiwara S, Kawamura K, Yubisui T (2000) Functional retinoid receptors in budding ascidians. *Dev Growth Differ* 42:1–8
- Kanayama T, Kobayashi N, Mamiya S, Nakanishi T, Nishikawa J (2005) Organotin compounds promote adipocyte differentiation as agonist of the peroxisome proliferators-activated receptor γ /retinoid X receptor pathway. *Mol Pharmacol* 67:766–774
- Kostrouch Z, Kostrouchova M, Love W, Jannini E, Piatigorsky J, Rall JE (1998) Retinoic acid X receptor in the diploblast, *Tripedalia cystophora*. *Proc Natl Acad Sci USA* 95:13442–13447
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- le Maire A, Grimaldi M, Roecklin D, Dagnino S, Vivat-Hannah V, Balaguer P, Bourguet W (2009) Activation of RXR-PPAR heterodimers by organotin environmental endocrine disruptors. *EMBO Rep* 10:367–373
- Mangelsdorf DJ, Evans RM (1995) The RXR heterodimers and orphan receptors. *Cell* 83:841–850
- Mangelsdorf DJ, Borgmeyer U, Heyman RA, Zhou JY, Ong ES, Oro AE, Kakizuka A, Evans RM (1992) Characterization of three RXR genes that mediate the action of 9-*cis*-retinoic acid. *Genes Dev* 6:329–344
- Matthiessen P, Gibbs PE (1998) Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in mollusks. *Environ Toxicol Chem* 17:37–43
- Matthiessen P, Reynoldson T, Billingham Z, Brassard DW, Cameron P, Chandler GT, Davies IM, Horiguchi T, Mount DR, Oehlmann J, Pottinger TG, Sibley PK, Thompson A, Vethaak AD (1999) Field assessment for endocrine disruption in invertebrates. In: de Fur PL, Ingersoll C, Tattersfield L (eds) Endocrine disruption in invertebrates: endocrinology, testing, and assessment. SETAC Press, Pensacola, Florida, pp 199–270
- Morris-Kay GM (1997) Retinoids in mammalian embryonic development. In: Sherbet GV (ed) Retinoids: their physiological function and therapeutic potential. JAI Press, Greenwich, pp 79–93
- Nagatomo K, Fujiwara S (2003) Expression of Raldh2, Cyp26 and Hox-1 in normal and retinoic acid-treated *Ciona intestinalis* embryos. *Gene Expr Patterns* 3:273–277
- Nagatomo K, Ishibashi T, Satou Y, Satoh N, Fujiwara S (2003) Retinoic acid affects gene expression and morphogenesis without upregulating the retinoic acid receptor in the ascidian *Ciona intestinalis*. *Mech Dev* 120:363–372
- Nishikawa J, Mamiya S, Kanayama T, Nishikawa T, Shiraishi F, Horiguchi T (2004) Involvement of the retinoid X receptor in the development of imposex caused by organotins in gastropods. *Environ Sci Technol* 38:6271–6276
- Oberdörster E, McClellan-Green P (2000) The neuropeptide APGWamide induces imposex in the mud snail, *Ilyanassa obsoleta*. *Peptides* 21:1323–1330
- Redfern CPF (1997) Molecular mechanisms of the retinoid function. In: Sherbet GV (ed) Retinoids: their physiological function and therapeutic potential. JAI Press, Greenwich, pp 35–77
- Ronis MJJ, Mason AZ (1996) The metabolism of testosterone by the periwinkle (*Littorina littorea*) *in vitro* and *in vivo*: effects of tributyltin. *Mar Environ Res* 42:161–166
- Smith BS (1971) Sexuality in the American mud snail, *Nassarius obsoletus* Say. *Proc Malacol Soc Lond* 39:377–378
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354
- Wiens M, Batel R, Korzhev M, Muller WE (2003) Retinoid X receptor and retinoic acid response in the marine sponge *Suberites domuncula*. *J Exp Biol* 206:3261–3271