Masculinization of Female Gastropod Mollusks Induced by Organotin Compounds, Focusing on Mechanism of Actions of Tributyltin and Triphenyltin for Development of Imposex

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Imposex, the superimposition of male-type genital organs (penis and vas deferens) on female gastropods, is cause-specific and induced by low concentrations of certain organotins, such as tributyltin (TBT) and triphenyltin (TPT) from antifouling paints. Reproductive failure may be observed at severely affected stages. The history of imposex study and the legislation of organotins are summarized. The present statuses of gastropod imposex and organotin pollution in Japan, together with endocrine disruption observed in abalones, are also summarized, on the basis of the results of the field studies of the rock shell (Thais clavigera) conducted at 174 locations along the Japan coast between January 1999 and November 2001, the histopathological and analytical chemical studies of the ivory shell (Babylonia japonica), and both field and laboratory studies of the abalone (Haliotis madaka and H. gigantea). The fundamental knowledge of the endocrinology of gastropod mollusks is briefly described. Four hypotheses, such as that of aromatase-inhibition, regarding the induction mechanism of imposex induced by organotins in gastropods are reviewed. Finally, a new hypothesis that states that retinoid X receptor (RXR) plays an important role in inducing the development of imposex, i.e., the differentiation and growth of male-type genital organs in female gastropods, is reviewed and discussed to clarify the entire mode of action of TBT or TPT in the development of imposex in gastropods.

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1. Introduction

Certain environmental chemicals could cause the feminization of males and/or the masculinization of females in organisms, and such phenomena are generally called endocrine disruptions.\(^{(1)}\) The current status of the 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)\(^{(2)}\). Here, the author will review the masculinization of female gastropod mollusks, i.e., imposex, in terms of the basic biology and induction mechanism of imposex as well as its current status in gastropods.

The first report on masculinized female gastropod mollusks was made by Blaber\(^{(3)}\), describing a penis-like outgrowth behind the right tentacle in spent females of the dog-whelk *Nucella lapillus* around Plymouth, U.K. The term imposex, however, was coined by Smith\(^{(4)}\) 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.\(^{(5)}\) Reproductive failure may occur in females with severe imposex, resulting in population decline or even mass extinction.\(^{(6,7)}\) In some species, imposex is typically induced by tributyltin (TBT) and triphenyltin (TPT), chemicals released from antifouling paints used on ships and fishing nets.\(^{(8–12)}\)

As of 2004, worldwide, approximately 150 gastropod species have been reported to be affected by imposex\(^{(13–20)}\); 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.\(^{(15,16)}\)

Regarding Japanese gastropods, at least 39 species (7 mesogastropods and 32 neogastropods) have been found to be affected by imposex among the 69 species examined.\(^{(16,21)}\) Although imposex has been observed mostly in shallow-water species in previous surveys, detailed studies of species living at a depth of 200 m or more must be conducted because of the latest finding of imposex in Alabaster False Tun (*Galeoocorys leucodoma*) trawled from depths of 200–250 m off the Atsumi Peninsula in 1999.\(^{(21)}\)

In previous studies, the incidence or severity of imposex has been examined, the use of certain gastropod species as biological indicators of TBT contamination has been investigated, and TBT contamination using gastropods has been surveyed. However, only a few studies have shown evidence of the population-level effects of reproductive failure due to imposex on the basis of either morphological or histological methods.\(^{(5,6,21–29)}\)

TBT and TPT compounds (TBTs and TPTs) have been used worldwide in antifouling paints for ships and fishing nets since the mid-1960s, although lower amounts of TPTs have been used.\(^{(25,30)}\) In Japan, the production, importation and use of TBTs and TPTs have been strictly regulated by legislation and government administrative guidance since 1990. These activities were reported to have been completely stopped by 1997, although evidence suggests illegal TBT use in antifouling paints in some areas.\(^{(21,25)}\) The International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention) was adopted to enforce a worldwide ban on TBT and TPT during the International Maritime Organization (IMO) Convention in October 2001,\(^{(31)}\) although the ban has not yet come into effect.
2. Current Status of Imposex and Contamination by Organotins in Several Gastropods from Japan

2.1 Imposex in rock shell (T. clavigera) and organotin pollution in Japan

Among rock shell (T. clavigera) samples collected between January 1999 and November 2001 from 174 locations along the Japan coast, imposex was observed in 166 locations whereas no or rare cases were found in the remaining eight locations. The percentage occurrence of imposex was still as high as or close to 100% in approximately half of the locations surveyed. It is expected that spawning obstruction occurs in more than half the population of females when relative penis length (RPL) index exceeds 40, on the basis of the relationships among RPL index, vas deferens sequence (VDS) index and the percentage occurrence of oviduct (vulva) blockage in females. Among the 174 locations, RPL index values exceeding 40 were found in 41 locations. High values of RPL and VDS indices were generally observed in the western part of Japan. Compared with the results of a survey conducted previously (from 1996 to 1999), the indices seemed to have decreased but remained almost unchanged in some locations.

TPT concentrations in the tissues of the rock shell showed a decrease over time but varied distinctly among locations; relatively high pollution levels in a few locations were detected. Decreases in TBT concentrations were also distinct in general but the degree of decrease was lower than for those in TPT concentrations. Changes in concentrations over time were not observed in several locations. An increase in the concentrations of TBT was observed in two locations near fishing ports.

2.2 Impact of organotin pollution on ivory shell (B. japonica) populations

The histopathological examination of the gonads and the chemical analysis of organotin compounds in the tissues of ivory shell (B. japonica), using 135 individual shells (43 males and 92 females) sampled monthly between December 1988 and November 1989 in Prefecture A, Japan, showed that imposex occurred in approximately 80–90% of the B. japonica specimens examined, and that both penis and vas deferens were found to be well developed; no oviduct blockage by vas deferens formation was observed. Ovarian spermatogenesis and suppressed ovarian maturation were also observed in imposex-exhibiting females, although no histopathological abnormalities were observed in males. The tissue distributions of organotin compounds (i.e., TBT, TPT and their metabolites) were different from those of butyltins and phenyltins: a remarkably high accumulation of TBT was observed in the ctenidium, osphradium, and heart, whereas high concentrations of TPT were detected in the ovary and digestive gland. More than one-third and one-half of the total TBT and TPT, respectively, accumulated in the digestive glands of both males and females. Both TBT and TPT concentrations in the gonads were positively correlated with penis length in females. These findings strongly suggested that reproductive failure in adult females accompanied by imposex, possibly induced by TBT and TPT from antifouling paints, might have caused the marked decrease in B. japonica populations in Japan.

2.3 Endocrine disruption in abalones (Haliotis madaka and H. gigantea) and its possible impact on abalone populations

The total catch of abalone has been decreasing in Japan since the 1970s. Various studies have been conducted to determine the causal factors for the decrease in total catch but they
still remain unresolved. With the hypothesis that organotins (i.e., TBT and TPT) might also cause reproductive failure in abalone populations, a preliminary survey was started in 1994, and then the gonadal maturity of the giant abalone (*Haliotis madaka*) was histologically examined from September 1995 to November 1996, with area B selected as a reference area, which was considered to be relatively healthy in terms of catches and other characteristics, and with area C selected as the impacted area, where drastic decreases in the amount of catches were observed and the ratio of artificially raised and released seeds to the total catch was very high (i.e., reproduction in the natural abalone populations was suspected to be very low).\(^{(33)}\)

The results revealed that synchronous sexual maturation was observed in both females and males in area B whereas it was not observed in the giant abalone population in area C, implying a low fertilization rate of the giant abalone population in area C.\(^{(33)}\) Moreover, eleven out of 54 females were masculinized (as evidenced by ovarian spermatogenesis; the incidence was approximately 20%) in area C although no hermaphrodites were observed in area B.\(^{(33)}\) The observation of approximately 20% hermaphrodites (masculinized females) in the giant abalone population in area C is suggested to be abnormal because abalone is known to be dioecious. This masculinization of female abalones was similar to the imposex observed in the rock shells (*T. clavigera*) and ivory shells (*B. japonica*).\(^{(33)}\) As imposex in neogastropods is known to be typically induced by certain organotins such as TBT and TPT, organotins were also suspected as causal agents for the masculinization of female giant abalones in area C. The tissue concentrations of organotins, determined by gas chromatography with a flame photometric detection (GC-FPD) system, in the giant abalones in area C were significantly higher than those in area B \((p<0.01)\).\(^{(33)}\) These were also observed in the *H. gigantea* population in area C, and such endocrine disruption and contamination by organotins in the abalone population in area C were still continuously observed in the survey from January 1998 to March 1999.\(^{(34)}\)

A 7-month *in situ* exposure experiment, using caged abalones (*H. gigantea*) from area B close to a dockyard in area C, resulted in significant accumulations of TBT and TPT in tissue \((p<0.01)\) and ovarian spermatogenesis in approximately 90% of females exposed to TBT and TPT on-site.\(^{(33)}\) No ovarian spermatogenesis was observed in the control specimen in area B.\(^{(33)}\) A two-month flow-through exposure of abalones (*H. gigantea*) to either TBT or TPT (nominal concentrations: 100 ng/l) caused significant spermatogenesis in the ovaries of exposed females.\(^{(35)}\) No significant histological changes were observed in the testes of exposed males.\(^{(35)}\) The microscopic features of the observed ovarian spermatogenesis are the same as those observed in the ivory shell (*B. japonica*) affected by imposex. Ovarian spermatogenesis and testicular development associated with imposex have also been observed in the rock shell (*T. clavigera*), ivory shell (*B. japonica*), and other Neogastropoda species.\(^{(22,27,28,33–36)}\) The findings indicate that masculinization (ovarian spermatogenesis) that can be considered similar to imposex occurs in abalones as an endocrine disruption phenomenon without the development of external genital organs such as a penis.\(^{(35)}\) Because remarkably high concentrations of TBT and TPT were observed in the head (including the central nervous system ganglia), compared with muscles concentrations, the accumulations of TBT and TPT in the head may disturb reproductive hormonal regulators through neuropeptides released from the affected ganglia.\(^{(35)}\) These neuropeptides may be one of the inducers of spermatogenesis in the abalone ovaries.\(^{(35)}\)
3. Mechanism of Action of Organotin Compounds for Development of Imposisx in Gastropods

3.1 Endocrinology of gastropod mollusks

Because of the lack of information on the basic biology of mollusks, the knowledge of reproductive physiology and/or endocrinology of gastropods has been very limited. Knowledge has been mainly obtained from certain species of Opisthobranchia (e.g., *Aplysia californica*) and Pulmonata (e.g., *Lymnaea stagnalis*); that is, several neuropeptides released from the visceral ganglia, cerebral ganglia, or the prostate gland of gastropods (e.g., *A. californica* and *L. stagnalis*) are egg-laying, ovulation, or egg-releasing hormones. Little knowledge of the reproductive physiology and/or endocrinology of Prosobranchia (including Archaeo-, Meso- and Neogastropoda), however, has been obtained.

Although LeBlanc et al. reviewed many studies and described that gastropods have both peptide and steroid hormones, it remains unclear 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. 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. 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*. 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 synthetic estrogen, ethynylestradiol, in the gonads indicated that contamination of the habitat of *B. japonica* had occurred. Similarly, contamination with other vertebrate-type sex steroids of the habitats of *T. clavigera* and *B. japonica* may have occurred.

Evidence for steroid-producing cells and synthetic/metabolic enzymes for steroid biosynthesis must be completely obtained to clarify the existence of vertebrate-type sex steroid hormones in gastropods. Although aromatase-like activity has been measured and reported in several gastropod species, the measured aromatase-like activity does not necessarily mean 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.

On the basis of a study of fully sequenced invertebrate genomes, homologues of estrogen receptors (ERs) and androgen receptors (ARs) have not been found in invertebrates. Thus, it remains unclear whether gastropods have ARs or ERs. Although ER-like cDNA was isolated from *A. californica* (Gastropoda: Opisthobranchia), it could not bind to estrogen and it was found that it was a constitutively activated transcription factor. ER-like protein was also isolated from the rock shell (*T. clavigera*), but it could not bind to estrogen either, and
it was also a constitutively activated transcription factor, similar to *A. californica* (Katsu, Iguchi and Horiguchi, unpublished data). Therefore, further studies are necessary to identify steroid receptors and clarify their functions in gastropods.

### 3.2 Mechanism of action of organotin compounds on development of imposex

Regarding the induction mechanism of imposex, several mechanisms have been proposed and they can be summarized as follows: 1) increased androgen levels, such as testosterone, due to aromatase inhibition by TBT;\(^{(49-51)}\) 2) inhibition of the excretion of sulfate conjugates of androgens by TBT;\(^{(52)}\) 3) disturbance of the release of penis morphogenetic/retrogressive factor from pedal/cerebropleural ganglia by TBT;\(^{(53)}\) 4) increase in the neuropeptide APGWamide level caused by TBT.\(^{(54,55)}\)

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.\(^{(49)}\) 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 advanced imposex symptoms in the gastropod *Bolinus brandaris*.\(^{(45)}\) Santos *et al.*\(^{(50)}\) suggested the involvement of an AR, besides aromatase inhibition, in the development of imposex in *N. lapillus*, although gastropods may not inherently have ARs.\(^{(47)}\) If gastropods also have ARs similar to those of 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.\(^{(56)}\)

There is a possibility that the results given in support of the testosterone excretion-inhibition hypothesis\(^{(52)}\) may reflect a phenomenon that is at least partly short-term and/or associated with acutely toxic TBT concentrations.\(^{(57)}\)

Several neuropeptides released from the visceral ganglia, cerebral ganglia, or the prostate gland of gastropods (*e.g.*, *A. californica* and *L. stagnalis*) are egg-laying, ovulation, or egg-releasing hormones.\(^{(37,38)}\) Féral and Le Gall\(^{(53)}\) 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 \(\mu g/l\)).\(^{(53)}\) The accumulation of TBT or TPT in the central nervous systems of *H. gigantea*,\(^{(35)}*N*. lapillus*,\(^{(50)}*T*. clavigera*\(^{(59)}\) indicates the potential for the toxic effects of TBT and TPT on neuroendocrine systems. Oberdörster and McClellan-Green\(^{(54,55)}\) 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 on the induction and/or promotion of the development of imposex, however, appears weak, on the basis of the experimental results of the incidences of imposex and penis growth.\(^{(54,55)}\)

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

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.\(^{6,21–25,27–29,33–36}\) 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.\(^{21}\)

Nishikawa \textit{et al.}\(^{60}\) proposed a unique mechanism of action of TBT or TPT on the development of imposex in gastropods, which was completely different from other mechanism already proposed for imposex induction. They showed that organotins (both TBT and TPT) bound to human retinoid X receptors (hRXRs) with high affinity and the injection of 9-cis retinoic acid (9-cis RA), the natural ligand of hRXRs, into female rock shells (\textit{T. clavigera}) induced the development of imposex. The cloning of an RXR homologue from \textit{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 9-cis RA and organotins.\(^{60}\) 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 of RXR gene expression, RXR protein content, immunohistochemical staining with an anti-RXR antibody, and time-course RXR gene expression after exposure to organotins with the rock shell (\textit{T. clavigera}) further support the hypothesis that RXR plays an important role in inducing the development of imposex caused by organotins in female gastropods (Nishikawa, Ohta and Horiguchi, manuscript in preparation). Further studies of histological, immunohistochemical, biochemical and molecular biological techniques are needed to elucidate the complete mechanism of action of TBT or TPT for the development of imposex in gastropods; this may involve a clarification of the 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, which could lead to the epidermal differentiation and proliferation of the penis and vas deferens formation, are initiated and promoted. A certain morphogenetic factor could be involved in the formation of the curved penis and vas deferens. It is also possible that other factors, such as certain neuropeptides, might be associated with the development of imposex through RXR gene expression induced in the head ganglia by exposure to organotins, if these factors are induced downstream of the RXR cascade.\(^{61}\)

\textbf{References}


