

Chapter 11

Reproductive Organ Development in the Ivory Shell *Babylonia japonica* and the Rock Shell *Thais clavigera*

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Abstract Results of histological examination of normal differentiation and development of the genital tract and gonad in the ivory shell *Babylonia japonica* (Buccinidae) are described. The formation of male-type genitalia (penis and vas deferens) in imposex-exhibiting females seems to mimic the normal development of male genitalia in prosobranch gastropods, on the basis of observations using a wild-caught 2-year-old specimen and laboratory-reared juveniles aged 0–24 months. Gonad differentiation was unclear before age 14 months but progressed after 16 months. Both sexes had a complete genital tract and mature gonads at 20 months. However, differentiation and development occurred earlier in females than in males. Development of the genital tract preceded gonad differentiation. Vas deferens morphogenesis in males resembled that in imposex-exhibiting females. Histological examination of the development of male genitalia in imposex-exhibiting female rock shells, *Thais clavigera* (Muricidae), using specimens from a wild population and tributyltin (TBT)-exposed females in the laboratory, allowed observation of a variety of vas deferens morphogenesis patterns. Taking into consideration observed results both from wild female specimens and from TBT-exposed females in the laboratory, the vas deferens sequence (VDS) index for *T. clavigera* has been proposed as VDS 1–6, which is a little different from that for *Nucella lapillus*. Comparison of the differentiation and development of male genitalia in normal males and imposex-exhibiting females among gastropod species implies it does not seem to be strictly regulated: relatively large variation in the differentiation and development of genitalia could occur among individuals, as well as among species of prosobranch gastropods.

Keywords Imposex • Ivory shell (*Babylonia japonica*) • Rock shell (*Thais clavigera*) • Vas deferens • Penis • Gonad • Differentiation • Morphogenesis • Tributyltin (TBT) • Triphenyltin (TPHT) • Retinoid X receptor (RXR)

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T. Horiguchi (ed.), *Biological Effects by Organotins*,
DOI 10.1007/978-4-431-56451-5_11

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Abbreviations

AFS	the International Convention on the Control of Harmful Anti-fouling Systems on Ships
DMSO	dimethyl sulfoxide
EDCs	endocrine-disrupting chemicals
H&E	hematoxylin and eosin
IMO	International Maritime Organization
OECD	Organization for Economic Co-operation and Development
9cRA	9- <i>cis</i> retinoic acid
RXR	retinoid X receptor
TBT	tributyltin
TBTCl	tributyltin chloride
TPhT	triphenyltin
VDS index	vas deferens sequence index

11.1 Introduction

Masculinized female gastropod molluscs were first reported by Blaber (1970), who described a penis-like outgrowth behind the right tentacle in spent females of the dog whelk, *Nucella lapillus*, around Plymouth, UK. The term “imposex,” however, meaning “imposed sexual organs,” was defined by Smith (1971) to describe the syndrome of superimposition of male genitalia, such as a penis and vas deferens, on female prosobranch gastropods. Currently, imposex is thought to be an irreversible syndrome (Bryan et al. 1986). In severe cases of imposex, reproductive failure may occur, resulting in population decline or mass extinction (Gibbs and Bryan 1986, 1996). Imposex in many species is induced by tributyltin (TBT) and triphenyltin (TPhT) released from antifouling paints on ships and fishing nets (Bryan et al. 1987, 1988; Gibbs et al. 1987; Horiguchi et al. 1995, 1997). The use of TBT- or TPhT-based antifouling paints was addressed by the International Convention on the Control of Harmful Anti-fouling Systems on Ships (AFS Convention), which was adopted by the International Maritime Organization (IMO) on 5 October 2001 (IMO 2001). According to the AFS Convention, all ships are prohibited from applying or reapplying organotin compounds as antifouling biocides after 1 January 2003, and by 1 January 2008 ships either will not use organotin compounds as antifouling biocides or such antifouling systems will be covered with a coating that prevents leaching into the environment. It took longer than expected for the AFS Convention to be ratified by member states, but it finally came into effect on 17 September 2008 (<http://www.imo.org/>). Continued monitoring is needed to protect marine and aquatic ecosystems from organotin pollution and to allow the systems to recover from its impacts.

On the other hand, it is necessary to establish useful testing methods to properly evaluate and strictly regulate harmful chemical substances, such as

endocrine-disrupting chemicals (EDCs), to protect and conserve ecosystems. OECD has established several test guidelines for wildlife (i.e., fish and crustaceans), but not yet for mollusks (OECD 2016). Although fundamental knowledge of the biology, including reproductive physiology and endocrinology, of mollusks is essential to develop and establish a useful test method to properly evaluate harmful chemical substances, such as EDCs, for valid legislation, less is known about molluscan basic biology (see Chaps. 8 and 9). For example, it is doubtful whether gastropod mollusks have vertebrate-type steroids as sex hormones (see Chap. 9). Also, the differentiation of gonads and accessory sex organs (i.e., genital tracts) is still obscure in mollusks. The histological development of the vas deferens and penis in *N. lapillus* exhibiting imposex was reported by Gibbs and Bryan (1986) and Gibbs et al. (1987). Less is known about imposex development, in terms of the general development of the vas deferens and penis, in other species, although there are a few reports describing the general scheme of imposex development in prosobranch gastropods (Stroben et al. 1995). In the case of *Thais clavigera*, this process remained unclear until recently, because extensive contamination by organotins (TBT and TPhT) throughout Japan (Horiguchi et al. 1994) meant that only specimens in the severe stages of imposex had been observed there. Horiguchi et al. (2012a), by histological analysis of less severely exposed specimens from a wild population and females exposed to TBT in the laboratory, finally revealed the steps in the development of the vas deferens and penis in the imposex-exhibiting female rock shell *T. clavigera*. They observed a variety of patterns of vas deferens morphogenesis in wild females of *T. clavigera*. The immature vas deferens, however, was observed only beneath or behind the penis, and no vas deferens was observed close to the vaginal opening (i.e., vulva) of the capsule gland in TBT-exposed female *T. clavigera*. This observation differed from the vas deferens formation observed in wild female *T. clavigera*, as well as in female *N. lapillus* (Gibbs et al. 1987). Considering the observations of both wild and TBT-exposed females of *T. clavigera* in the laboratory, the vas deferens sequence (VDS) index for *T. clavigera* was proposed as VDS 1–6 (Horiguchi et al. 2012a). This VDS index differs from that of *N. lapillus* (Horiguchi et al. 2012a), especially in the initial developmental stages of imposex. Thus, it is possible that the processes responsible for development of the vas deferens and penis in imposex-exhibiting female gastropods differ among gastropod species.

In the ivory shell *Babylonia japonica*, histopathological and analytical chemical studies strongly suggest that reproductive failure in adult females accompanying imposex, possibly induced by TBT and TPhT, could have caused a marked decline in *B. japonica* populations; this decline might be one factor behind the decrease in the total catch of *B. japonica* in Japan since the 1970s (Horiguchi et al. 2006). Here, I discuss female and male *B. japonica* to discern normal differentiation and development of the genital tract and gonad (Horiguchi et al. 2014); this will be useful in determining whether the formation of male-type genitalia in imposex-exhibiting females mimics the normal development of male genitalia in prosobranch gastropods.

11.2 Development of Genitalia in *Babylonia japonica*

A 2-year-old male *Babylonia japonica* was used as a specimen from the wild population, which had been produced as seed at the hatchery of the Tottori Prefectural Sea Farming Association (TPSFA) and then released; it was captured in trawling nets in Miho Bay, Japan (35°27'50.37" N, 133°21'01.84" E) in July 2010. Laboratory-reared *B. japonica* juveniles were also used for the study. Adult *B. japonica* were collected in Miho Bay and landed at Yodoe, Tottori, Japan, as part of the commercial fishery, and then reared at the TPSFA hatchery for seed production in tanks with flow-through ambient-temperature natural seawater from the Sea of Japan. Egg capsules deposited by adults were rinsed with distilled water and then moved to other tanks with flow-through seawater. Larvae hatched after approximately 3 weeks. Veliger larvae settled toward the bottom of the tanks within several days of hatching. Settled juveniles were fed minced Antarctic krill (*Euphausia superba*) every day. Juvenile *B. japonica* were reared at TPSFA for 6 months (i.e., from age 0 to age 6 months). Juveniles older than 6 months were reared at the National Institute for Environmental Studies (NIES) in acrylic aquaria (length, 90 cm; width, 45 cm; height, 45 cm) containing a magnetic-drive circulating pump with the filtration media composed of coral sand and pieces. The aquaria were filled with deep seawater collected from a depth of about 400 m in Suruga Bay, Japan. Water temperature was maintained at 23 ± 1 °C by a water temperature controller. These juveniles were also fed Antarctic krill (*E. superba*) every day. Concentrations of TBT and TPHT in both seawater systems were below the detection limit. Laboratory-reared juveniles were kept in tanks at TPSFA or aquaria at NIES from July 2009 to August 2011. *B. japonica* juvenile samples were collected at 6, 12, 14, 16, 18, 20, and 24 months after hatching. The number of juveniles sampled was 6, 10, 10, 17, 15, 12, and 10, respectively (Horiguchi et al. 2014). After removal of the shell, whole soft tissues were fixed in Gendré's fluid (Horiguchi et al. 2002). Tissues, including the presumptive penis-forming area behind the right tentacle and the gonads, were removed and embedded in paraffin. Serial sections were prepared using a rotary microtome, stained with hematoxylin and eosin (H&E), and observed under a light microscope for histological examination of *B. japonica* genitalia and gonads (Horiguchi et al. 2014).

Table 11.1 summarizes the development of reproductive organs in the ivory shell *B. japonica* (Horiguchi et al. 2014).

In the 2-year-old wild-caught male specimen from Miho Bay, the germinal epithelium was composed mainly of spermatogonia and spermatocytes (Fig. 11.1B). It had a small penis behind the right tentacle, where the vas deferens and its opening were evident (Fig. 11.1A) (Horiguchi et al. 2014).

Regarding laboratory-reared juveniles, in females aged 6 months, the area of ovarian tissue was very narrow and immature, seeming to consist mainly of germinal cords. Several germinal cords were scattered under the epidermis near the digestive gland; these were different in length and not connected with each other. One of the germinal cords had a tubular form and was connected to the

Table 11.1 Summary of reproductive development in the ivory shell *Babylonia japonica*

Males	Age to initiate	Age to complete
Differentiation of testis	16 months	20 months
Vas deferens formation	14 months	20 months
Penis formation	16 months	20 months
Copulation for fertilization	N.C. (>24 months)	–
Females		
Differentiation of ovary	16 months	20 months
Development of vagina, bursa copulatrix, and capsule gland	14 months	20 months
Development of albumen and sperm-ingesting glands	16 months	20 months
Spawning of egg capsules	18 months	–

Horiguchi et al. (2014)

N.C. not confirmed

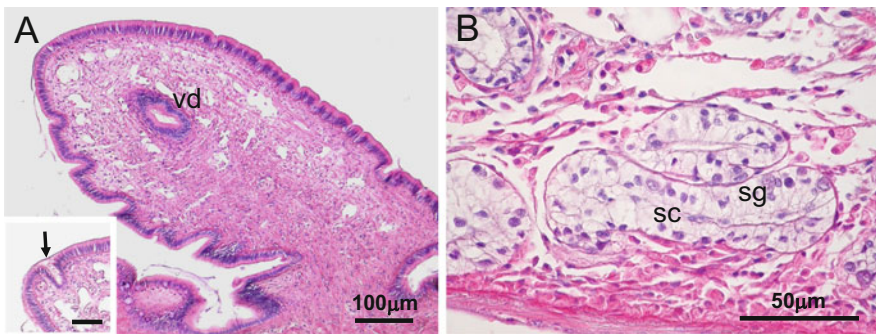


Fig. 11.1 Reproductive organs of a wild-caught male *Babylonia japonica*, age 2 years (Horiguchi et al. 2014). (A) Vas deferens (vd) in the penis. *Inset* (lower left) shows open vas deferens (*arrow*). *Bar* 50 µm. (B) Part of the testis containing spermatogonia (sg) and spermatocysts (sc) in the seminiferous tubules

immature oviduct. A few oogonia were observed (Fig. 11.2A). The vagina and capsule gland were not yet separated. Both vagina and capsule gland had single-layer cuboidal epithelium. Whether the vagina opened or not differed among individual specimens. In males aged 6 months, the testicular tissue area was very narrow and immature. It seemed to consist basically of germinal cords, some of which had partially formed into tubular shapes. A few spermatogonia were observed (Fig. 11.3A). Spermatogonia morphologically resembled oogonia, with polyhedral nuclei. No differentiation of the vas deferens was observed. The testicular duct consisted of transitional epithelium with scant and pale cytoplasm. The testicular duct morphologically resembled the oviduct (Fig. 11.4A). The terminus of the testicular duct, which was lined with a single layer of cuboidal cells, became a closed end near a shallow invagination of the epidermis near the kidney (Fig. 11.4B) (Horiguchi et al. 2014).

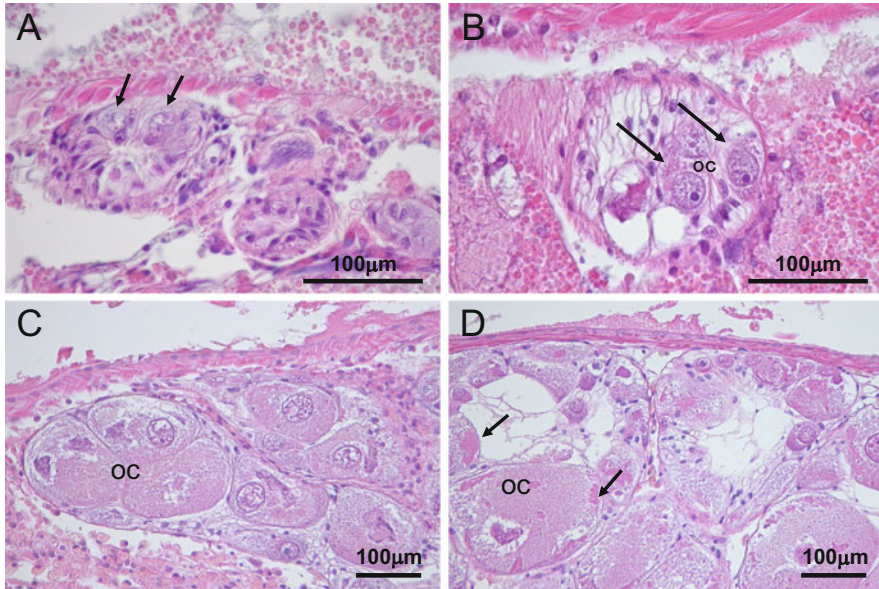


Fig. 11.2 Ovaries in female *Babylonica japonica*, age 6–16 months (Horiguchi et al. 2014). (A) Oogonia (arrows) in a 6-month-old specimen. (B) Oocytes at the early stage (oc) in a 12-month-old specimen (arrows). (C) Oocytes at the late stage in a 14-month-old specimen. (D) Mature oocytes containing eosinophilic granules (arrows) in a 16-month-old specimen

In females aged 12 months, the characteristics of the ovarian tissue were similar to those in 6-month-old females. The ovarian tissue area was narrow and immature close to the digestive gland. Many specimens showed only oogonia; rarely, immature oocytes as well as oogonia were observed (Fig. 11.2B). Germinal cords were lengthening and gradually connecting to each other. There was still no separation between the vagina and capsule gland (Fig. 11.5B). The vaginal opening still differed among individual specimens (Fig. 11.5A). One of the germinal cords formed a tube that was connected to the immature oviduct. Both vagina and capsule gland had single-layer cuboidal epithelium, but pseudo-stratification was observed in parts of the vagina and capsule gland. The oviduct had transitional epithelium with scant and pale cytoplasm (Fig. 11.5B). In males aged 12 months, the testicular tissue area was expanding compared with that in 6-month-old specimens. Germinal cords were tubular in form and were lengthening and thickening, although only spermatogonia were observed (Fig. 11.3B). The morphological difference between ovarian and testicular tissues was becoming clear. No differentiation of penis and vas deferens was observed. The testicular duct was similar to that in 6-month-old specimens (Horiguchi et al. 2014).

In females aged 14 months, the ovarian tissue was developing in a narrow area on the side opposite the digestive gland. Oocytes were observed in all specimens (Fig. 11.2C). One specimen contained oocytes that were almost mature, in which the ovarian tissue was developing well beyond the digestive gland. The vagina,

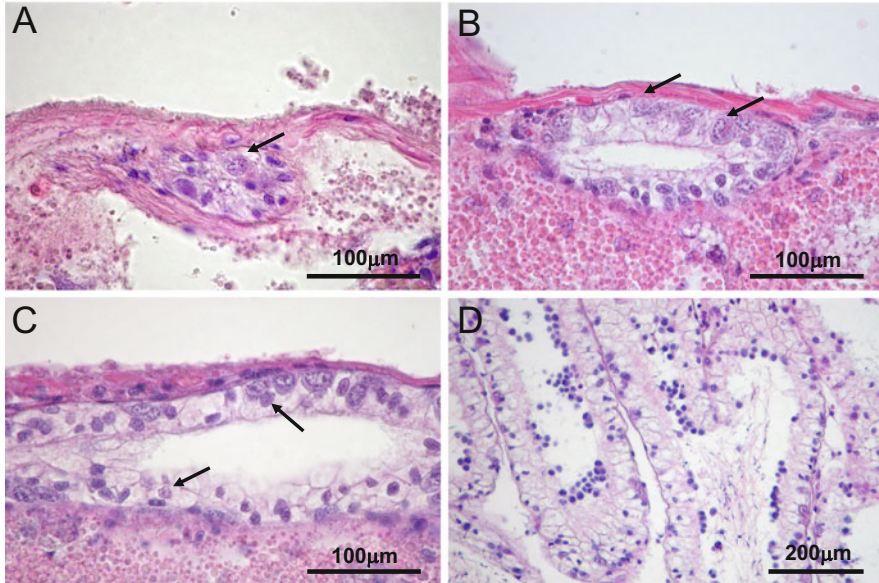


Fig. 11.3 Testes in male *Babylonia japonica*, age 6–16 months (Horiguchi et al. 2014). (A) Spermatogonium (arrow) in a 6-month-old specimen. (B) Multiple spermatogonia in a 12-month-old specimen (arrows). (C) Spermatocytes in a 14-month-old specimen (arrows). (D) All stages of spermatogenesis in a 16-month-old specimen

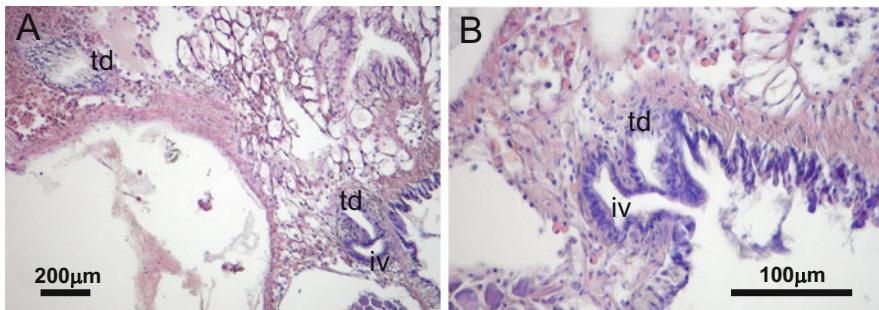


Fig. 11.4 Testicular duct near junction with the vas deferens in a male *Babylonia japonica*, age 6 months (Horiguchi et al. 2014). (A) Testicular duct (td) and invagination (iv) of epidermis near the kidney. (B) High-magnification view of invagination of the epidermis (iv)

bursa copulatrix, and capsule gland were also developing (Fig. 11.6.2). Differentiation of the sperm-ingesting gland as well as the albumen gland was recognized only in one female specimen (Fig. 11.6.1). The oviduct was connected to the lower genital duct. The vagina consisted of single-layer ciliated epithelium (Fig. 11.6.2A). An unknown secretory gland in part of the vagina consisted of single-layer columnar epithelium with eosinophilic cytoplasm (Fig. 11.6.2C). We preliminarily refer to this as the “vaginal gland.” Simple glands were arranged in

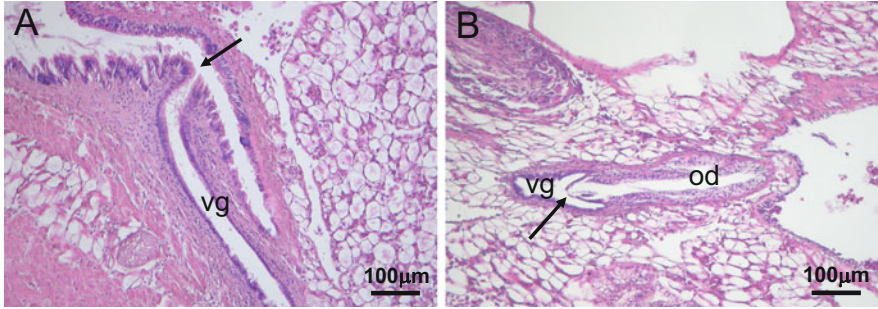


Fig. 11.5 Undifferentiated vagina and capsule gland in a female *Babylonia japonica*, age 12 months (Horiguchi et al. 2014). (A) Vagina/capsule gland (vg) and its opening (arrow). (B) Junction of vagina/capsule gland (vg) and oviduct (od)

cords forming the thick wall of the capsule gland (Fig. 11.6.2D). The superficial layer of the gland was composed of ciliated cells (Fig. 11.6.2D). Secretory cells contained many eosinophilic granules. The bursa copulatrix consisted of single- or double-layer cuboidal epithelium (Fig. 11.6.2B). In males aged 14 months, the testicular tissue area, where spermatocytes were observed, was developing in a narrow area close to the digestive gland (Fig. 11.3C). Despite the lack of penis formation, the vas deferens was gradually forming through invagination of the epidermis (Fig. 11.7B). The vas deferens, showing as a small depression, was incomplete and formed into a groove. Part of the groove of the discontinuous vas deferens became tubular (Fig. 11.7C). The vas deferens opened outside the mantle cavity near the kidney (Fig. 11.7D) (Horiguchi et al. 2014).

In females aged 16 months, the ovarian tissue area was developing widely, beyond the digestive gland. Mature or almost mature oocytes containing eosinophilic granules were observed in the ovary (Fig. 11.2D). The vagina, bursa copulatrix, capsule gland, sperm-ingesting gland, and albumen gland were completely differentiated (Fig. 11.8). The capsule gland was directly connected to the albumen gland (Fig. 11.8A, C). The capsule gland was thickening. The albumen gland consisted of branching folded epithelium, which consisted of columnar epithelium cells with basophilic granules in their cytoplasm (Fig. 11.8A–C). The sperm-ingesting gland consisted of single-layer epithelium, but its morphological features varied in part of the gland, where branching folded epithelium had formed. The epithelial cells of the sperm-ingesting gland were generally cuboidal, but columnar epithelium was present in the branching folded areas. The epithelial cells in the area connecting the sperm-ingesting gland and the capsule gland were ciliated (Fig. 11.8D). The oviduct close to the albumen gland was thick and consisted of ciliated epithelium, with branching folds. In contrast, the epithelial cells in the rest of the oviduct were columnar. Although the oviduct was connected to the albumen gland, we also observed a branch of the oviduct that opened into the mantle cavity leading outside the soft body. This part of the oviduct gradually thinned and consisted of transitional epithelium (Fig. 11.8B). In males aged

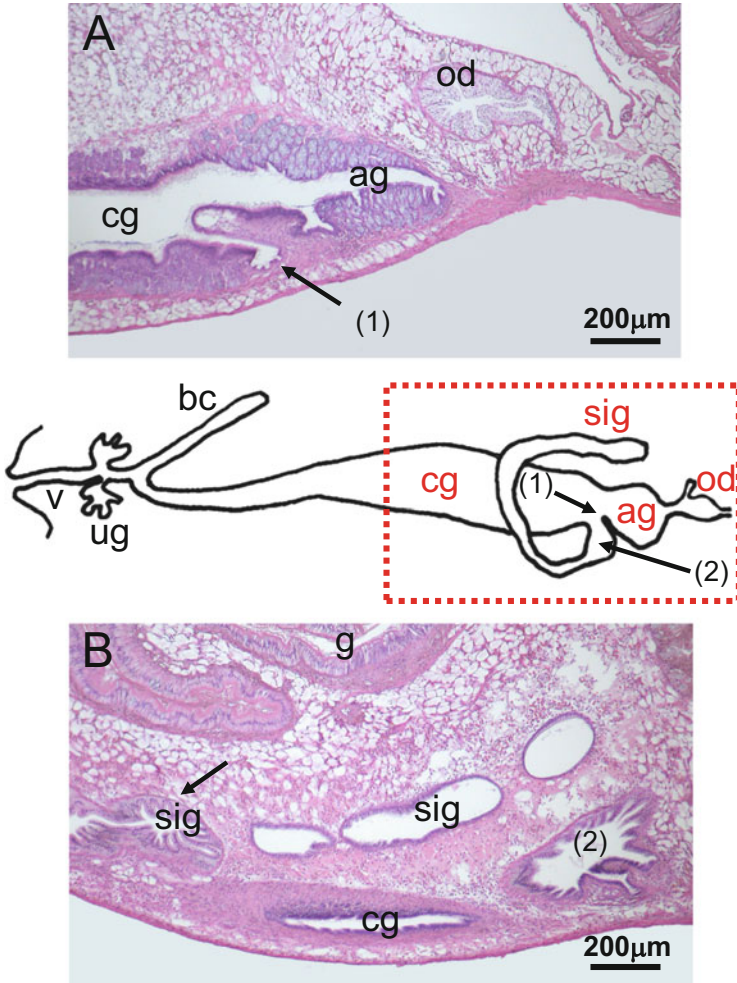


Fig. 11.6.1 Upper half of the genital tract of a female *Babylonia japonica*, age 14 months (Horiguchi et al. 2014). **(A)** Junction of oviduct (*od*), capsule gland (*cg*), and albumin gland (*ag*). Albumin gland consists of secretory cells with basophilic mucus. Sperm-ingesting gland opens to capsule gland (*arrow 1*). **(B)** Gut (*g*), capsule gland, and sperm-ingesting gland (*sig*). Portions of the sperm-ingesting gland are lined with branching fold epithelium (*arrow* and “2”)

16 months, the testicular tissue area was developing and spreading beyond the digestive gland. Spermatocytes and spermatids were generally observed in the testis of male specimens. Spermatozoa were observed in the testis of only one male, whereas the other one male had only spermatogonia in a narrowly developing testis (Fig. 11.3D). A protuberance for penis formation was observed behind the right tentacle in almost all male specimens, but invagination of the vas deferens into the penis was observed in only one male (Fig. 11.9B). Although the vas deferens was observed in the upper area (i.e., close to the prostate gland) in all male specimens,

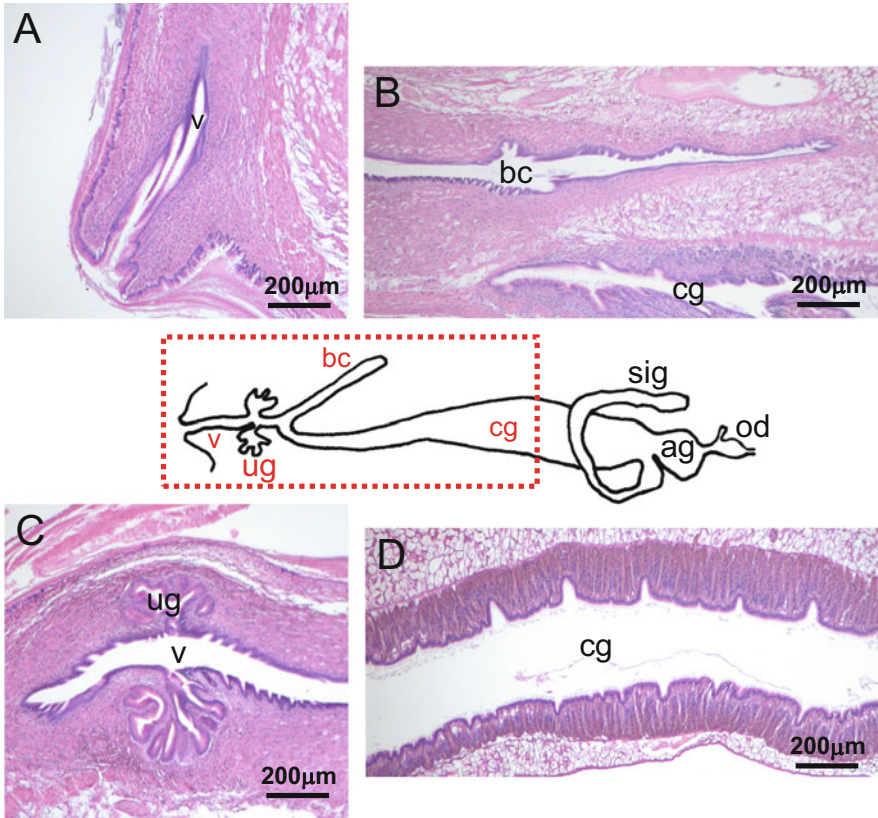


Fig. 11.6.2 Lower half of the genital tract of a female *Babylonia japonica*, age 14 months (Horiguchi et al. 2014). (A) Vagina (*v*) and vaginal orifice. (B) Bursa copulatrix (*bc*) and lower part of capsule gland (*cg*). (C) Unknown gland (*ug*) opening into the vaginal lumen. (D) Capsule gland consisting of a wall with simple glands

the vas deferens was not continuous in all specimens (Fig. 11.9). The epithelium of the vas deferens was lined with ciliated cells (Fig. 11.9C). The connection between the vas deferens and testicular duct varied considerably among specimens (Fig. 11.9D). It seems that the upper part of the vas deferens was formed through the invagination of epithelial cells and connected to the testicular duct close to the kidney (Fig. 11.9D). The duct then seemed to extend toward the penis behind the right tentacle, parallel to the rectum, to form the lower genital duct (i.e., vas deferens). Expansion of the duct seemed to result from the invagination of epithelial cells or fusion of the epithelial groove to form the duct structure; this differed from duct (i.e., vas deferens) formation from the opposite side (i.e., the area close to the penis behind the right tentacle). The testicular duct opened to the uppermost area of the vas deferens invagination at almost the same time as when the uppermost area of the vas deferens closed. In contrast, the vas deferens close to the penis seemed to form through invagination of the epithelium (Horiguchi et al. 2014).

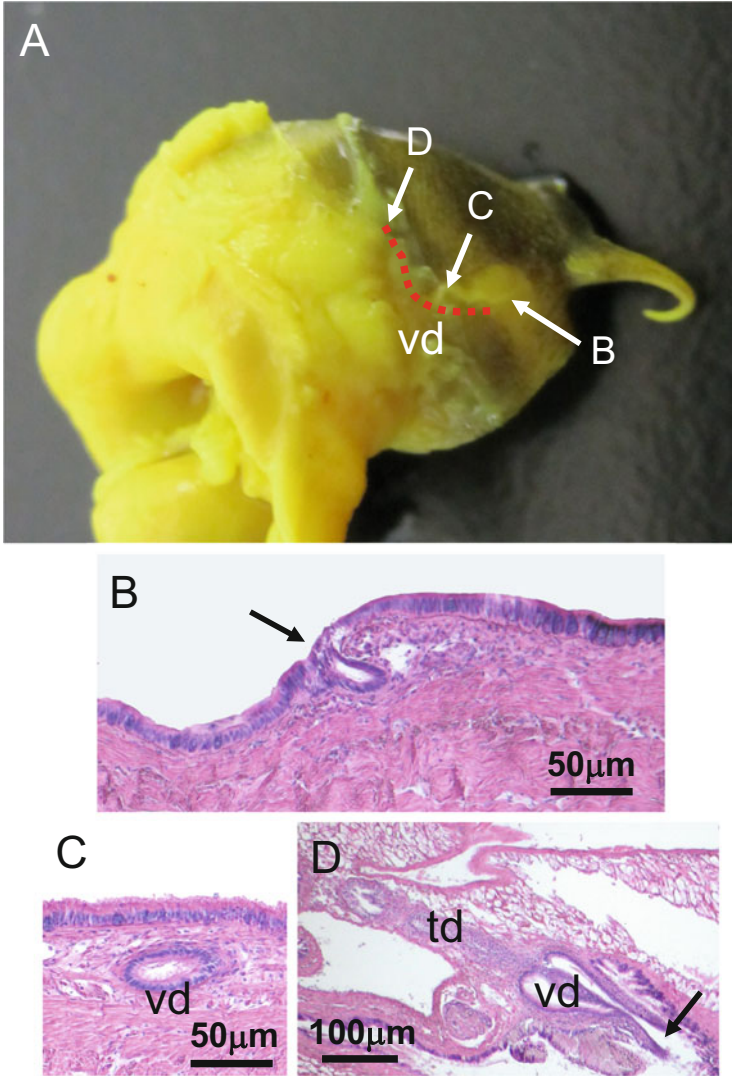


Fig. 11.7 Formation of the reproductive tract in a male *Babylonia japonica*, age 14 months (Horiguchi et al. 2014). (A) Appearance of soft body removed from the shell. Red dashed line indicates discontinuous vas deferens. (B) Invagination of vas deferens in penis-forming area (arrow). (C) Discontinuous vas deferens (vd) in cross section. (D) Junction area of vas deferens (vd) and testicular duct (td), showing opening to mantle cavity

In females aged 18 months, the ovarian tissue area was developing far beyond the digestive gland and had matured more than in 16-month-old specimens. The ovary contained many mature oocytes, which contained eosinophilic granules. Vagina, bursa copulatrix, capsule gland, sperm-ingesting gland, and albumen gland were completely differentiated. The oviduct was directly connected to the albumen gland. Other

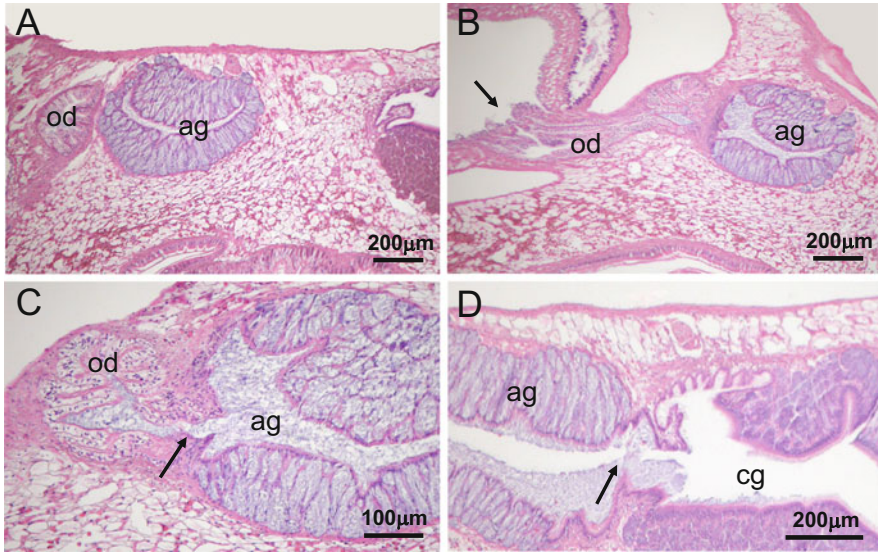


Fig. 11.8 Upper genital tract of a female *Babylonina japonica*, age 16 months (Horiguchi et al. 2014). (A) Oviduct (*od*) close to albumen gland (*ag*), which is lined with branching fold epithelium. (B) Branch of oviduct opening into the mantle cavity leading outside the body (*arrow*). (C) Junction of oviduct (*od*) and albumen gland (*ag*) (*arrow*). (D) Junction of albumen gland (*ag*) and capsule gland (*cg*) (*arrow*)

characteristics of the ovary and female accessory sex organs (i.e., genital tract) were the same as those observed in 16-month-old specimens. Females at age 18 months were observed to spawn and lay eggs (unfertilized) in aquaria at the NIES laboratory. In males aged 18 months, testicular tissue was more developed than in 16-month-old specimens, and half the specimens had spermatozoa in the testis. There were, however, large differences in testicular maturation among the male specimens. In mature males with spermatozoa in their testis, formation of the vas deferens was completed from the closed to open condition during development of the penis protuberance. In contrast, in male specimens with incomplete maturation of the testis, formation of the penis and vas deferens was incomplete and discontinuous (Horiguchi et al. 2014).

In females aged 20 months, the ovarian tissue was mature. The genital tract was completely developed: the vagina, bursa copulatrix, capsule gland, sperm-ingesting gland, and albumen gland were completely connected to each other. Histological features of the ovary and female genital tract were the same as those in 16-month-old specimens. In males aged 20 months, the testicular tissue was mature. The genital tract was completely developed: the testicular duct, vas deferens, and penis were completely connected to each other. However, spermatozoa were not observed in the vas deferens. The histological features of the testis and male genital tract were the same as those in 16-month-old specimens (Fig. 11.10) (Horiguchi et al. 2014).

In females aged 24 months, the ovarian tissue was mature. The genital tract was completely developed: the vagina, bursa copulatrix, capsule gland, sperm-ingesting

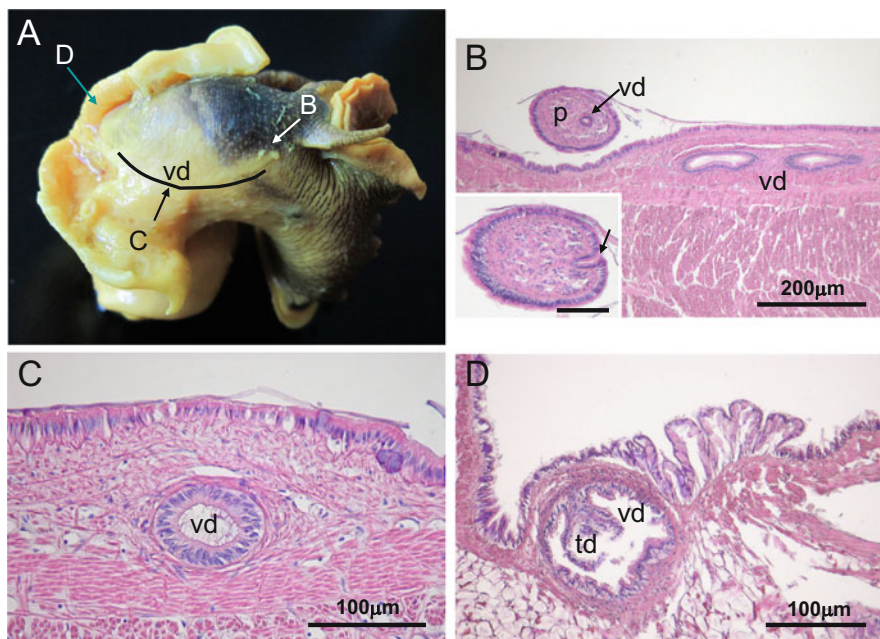


Fig. 11.9 Formation of the reproductive tract in a male *Babylonia japonica*, age 16 months (Horiguchi et al. 2014). (A) Appearance of soft body removed from the shell. *Black solid line* indicates the continuous (i.e., completely formed) vas deferens. (B) Vas deferens (vd) and adjacent penis (p). Inset (*lower left*) shows orifice of vas deferens in the penis of the same specimen (*arrow*). Bar 100 μm . (C) Vas deferens with ciliated epithelium in the body. (D) Junction of testicular duct (td) and vas deferens. Testicular duct is open into the vas deferens

gland, and albumen gland were completely connected to each other. Histological features of the ovary and female genital tract were the same as those in 16-month-old specimens. In males aged 24 months, the testicular tissue was mature. A few males appeared to have released sperm, judging from the histological features of their testis. The genital tract was completely developed. The testicular duct, vas deferens, and penis were completely connected to each other, but no spermatozoa were observed in the vas deferens in any of the male specimens. Although the vas deferens was open, penis size was still small (average penis length, 0.55 mm) (Horiguchi et al. 2014).

To understand the induction of imposex in prosobranch gastropods by organotin compounds, it is necessary to examine and understand in detail the normal processes of the genital tract and gonad differentiation and development. Because the planktonic stage of *B. japonica* is estimated to last approximately 4 to 5 days (Hamada et al. 1988, 1989), it would be easy to maintain and raise veliger larvae in the laboratory. Moreover, the methodology for hatchery production of *B. japonica* seed had been established since the 1980s (Kajikawa et al. 1983). Therefore, *B. japonica* is useful as a target species for research on differentiation and development of the genital tract and gonad (Horiguchi et al. 2014).

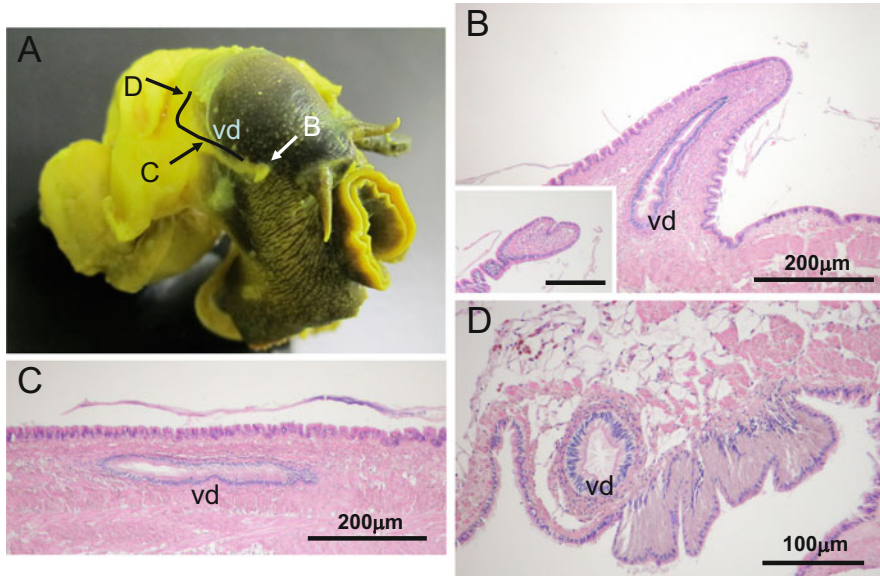


Fig. 11.10 Formation of the reproductive tract in a male *Babylonia japonica*, age 20 months (Horiguchi et al. 2014). (A) Appearance of soft body removed from the shell. *Black solid line* represents the completed vas deferens. (B) Vas deferens (*vd*) in the penis. Inset (*lower left*) shows the orifice of the vas deferens in the tip of penis of the same specimen. *Bar* 100 μ m. (C) Vas deferens (*vd*) in the body. (D) Vas deferens (*vd*) near the junction with the testicular duct

As described here and summarised in Table 11.1, the development of the *B. japonica* genital tract precedes differentiation of the gonad: this is the opposite of the sequence in vertebrates such as mammals (Gilbert 2006; Jost et al. 1973). This observation suggests that the regulatory mechanisms of endocrinological or reproductive organs and their functions differ between gastropods and vertebrates. In this regard, recent critical reviews of the presence of functional receptors for steroids and of enzymes for steroid synthesis or metabolism (Horiguchi 2009; Scott 2012, 2013, as well as Chap. 9), have pointed out that it is doubtful whether gastropod mollusks inherently have vertebrate-type steroids as sex hormones (Horiguchi et al. 2014).

Observations of a 2-year-old wild-caught male suggest that it takes about 2 years for complete development of the genital tract (i.e., testicular duct, vas deferens, and penis) and the mature testis. This finding does not contradict observations that laboratory-reared males at age 20 months and much older had a complete genital tract (i.e., testicular duct, vas deferens, and penis) and a mature testis (Horiguchi et al. 2014).

Differentiation and subsequent development of the genital tract and gonad seem to occur earlier in females than in males, an observation supported by the finding that 18-month-old females spawned and deposited eggs (unfertilized) in aquaria at the NIES laboratory. Males at the same age seem unable to copulate and fertilize eggs because of small penis size, incomplete vas deferens, and immature testis (Horiguchi et al. 2014).

The retinoid X receptor (RXR) could be mediating molecular mechanisms of the differentiation, proliferation, and morphogenesis of male genitalia in male and imposex-exhibiting female prosobranch gastropods (Nishikawa et al. 2004; Castro et al. 2007; Horiguchi et al. 2007, 2008, 2010a, b; Sternberg et al. 2008; Urushitani et al. 2011). Thus, development of a specific antibody for *B. japonica* RXR could provide useful information about when and where RXR expression is observed in the tissues of juvenile *B. japonica* under normal development. Laboratory experiments exposing *B. japonica* to TBT or TPhT over approximately 2 years, and using molecular, biochemical, and immunohistochemical techniques, could provide detailed information about the expression of mRNA for RXR and the presence of RXR protein during development under organotin exposure. The results of such studies should help clarify the mechanism of imposex induction by TBT and TPhT (Horiguchi et al. 2014).

11.3 Comparison of *Thais clavigera* and *Babylonia japonica*: The Formation of Male-Type Genitalia in Imposex-Exhibiting Females Mimics the Normal Development of Male Genitalia, with Difference Among Species

Various histological characteristics indicative of the initial stages of imposex were observed in females from a wild *Thais clavigera* population in Hiraiso, Japan (Horiguchi et al. 2012a) (Figs. 11.11 and 11.13). Unidentified aggregated cells, which may have been differentiating into a penis, and invagination of the epidermal tissue toward the formation of the vas deferens were observed in the presumptive penis-forming area of female *T. clavigera* (Horiguchi et al. 2012a) (Fig. 11.11A, B). In a female with a tiny penis, the epidermal tissue behind the penis was making an invagination, which was elongating into the penis to form an initial stage of the vas deferens (Fig. 11.12). However, this was a blind duct without any opening into the penis (Fig. 11.12A). Moreover, a variety of morphogenesis patterns of the vas deferens were observed in female *T. clavigera* specimens from a wild population in Hiraiso (Fig. 11.13). They are summarised as follows: (1) the invagination of the epidermal tissue toward the formation of the vas deferens occurs at almost the same time as a protuberance is formed in the presumptive penis-forming area behind the right tentacle of female *T. clavigera*; (2) the initial vas deferens is formed by the invagination of the epidermal tissue, followed by the extension and connection of the blind duct; (3) the invagination of the epidermal tissue toward the formation of the vas deferens occurs at several locations between the vaginal opening (i.e., vulva) of the capsule gland and penis, and then the vas deferens beneath the penis extends toward the tip of penis; and (4) the penis is differentiated and formed by unidentified aggregated cells in the epidermal tissue (Horiguchi et al. 2012a).

The five female rock shells that were removed from each group of flow-through exposure experiments, using TBT (exposure to TBTCI and a control group with

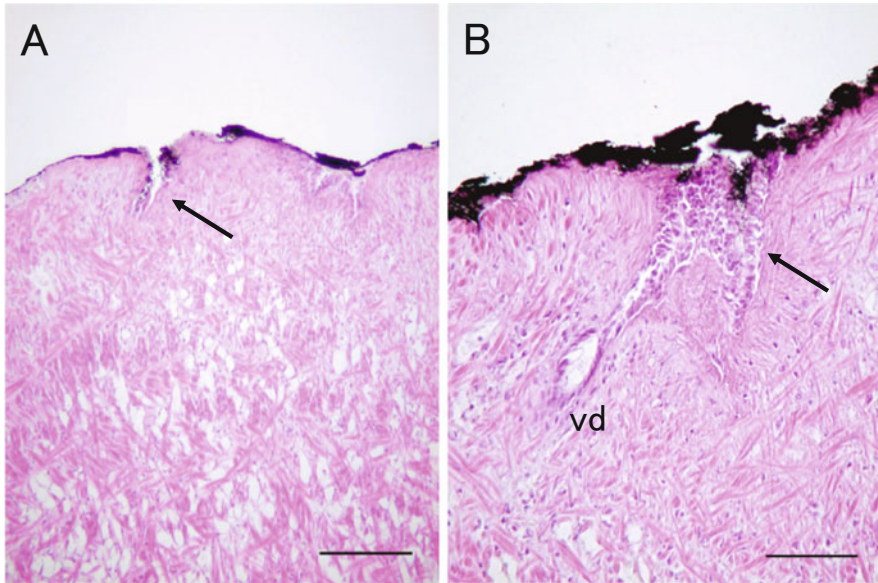


Fig. 11.11 Presumptive penis-forming area behind the right tentacle of a wild female *Thais clavigera* (Horiguchi et al. 2012a). *vd* vas deferens. The epidermis of the penis-forming area was marked with India ink after fixation. Note an invagination of the epidermal tissue (*arrow*), which will lead toward vas deferens formation (**A**), and unidentified aggregated cells (*arrow*), which are possibly differentiating into a penis (**B**). *Bar* 50 μ m

acetone/DMSO) after each of 5, 7, 12, and 24 days of the experiment were histologically examined under a light microscope to elucidate the processes of development of the vas deferens and penis during the initial stages of imposex in *T. clavigera*. After 5 days of TBT exposure, the five selected female specimens consisted of four imposex-exhibiting females and an apparently normal female. One female had an immature vas deferens (Fig. 11.14A) despite having no protuberance in the presumptive penis-forming area behind the right tentacle. In the other four females, however, no vas deferens (i.e., invagination of the epidermal tissue) was observed. After 7 days of TBT exposure, the five selected female specimens consisted of three imposex-exhibiting females and two apparently normal females. One female had an immature vas deferens as well as a protuberance in the presumptive penis-forming area behind the right tentacle (Fig. 11.14B). The vas deferens observed was not close to the vaginal opening of the capsule gland, but was behind a protuberance considered to be an initial stage of penis formation behind the right tentacle (Fig. 11.14B). No other females, however, displayed any invagination of the epidermal tissue, which would indicate immature vas deferens formation, after 7 days of TBT exposure. After 12 days of TBT exposure, the five selected female specimens consisted of two imposex-exhibiting females and three apparently normal females. Histological examination showed that the three normal-looking specimens had neither a penis nor vas deferens and that the two remaining

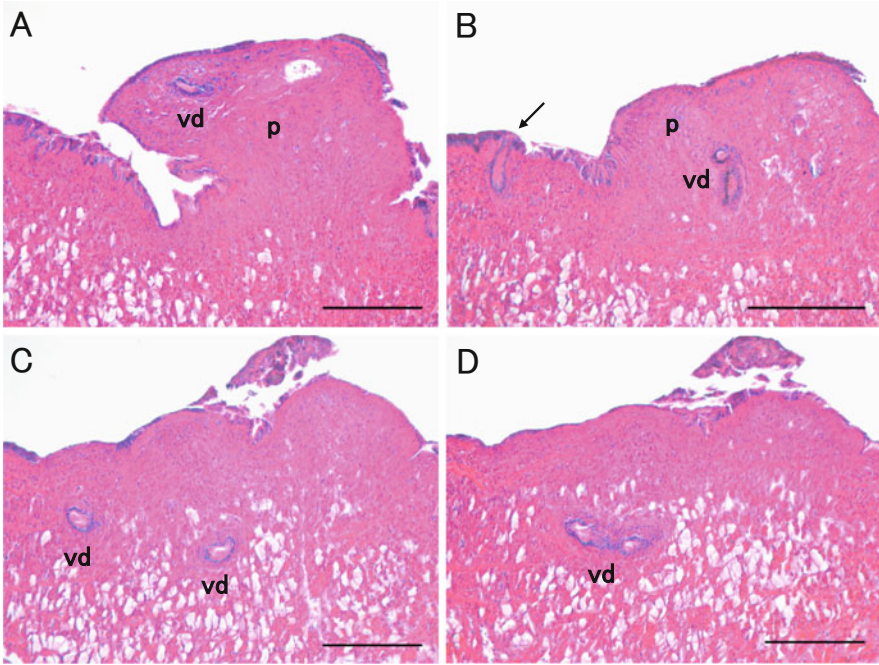


Fig. 11.12 Formation of the vas deferens behind the tiny penis of a wild female *Thais clavigera* (Horiguchi et al. 2012a). *p* penis, *vd* vas deferens. An invagination of the epidermal tissue (*arrow*) is visible behind the penis, forming the vas deferens (**B**). The vas deferens elongates into the penis (**C** and **D**), but it is a blind duct without any opening (**A**). *Bar* 100 μ m

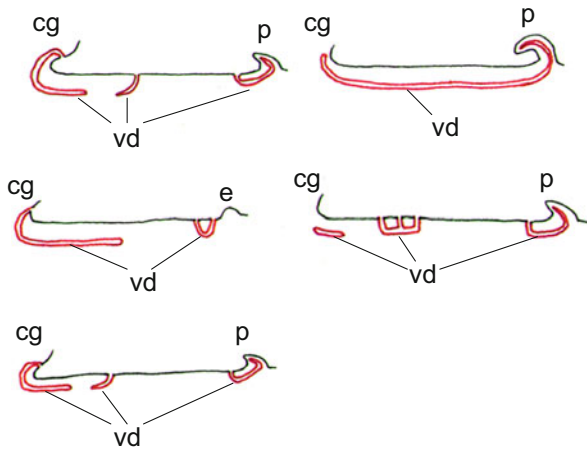


Fig. 11.13 A variety of patterns of vas deferens morphogenesis observed in wild females of *Thais clavigera* (Horiguchi et al. 2012a). *cg* capsule gland, *e* elliptical protuberance, *p* penis, *vd* vas deferens

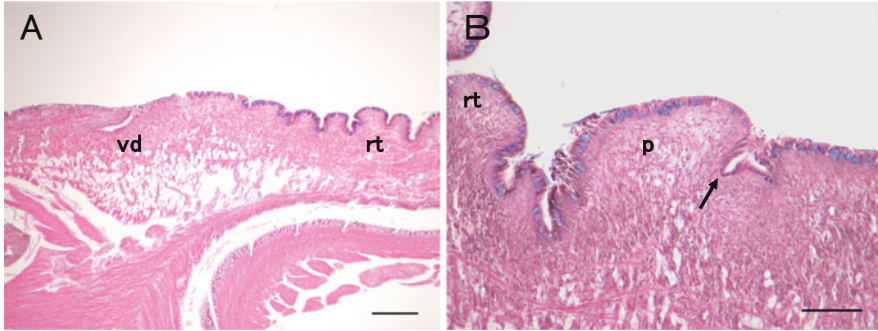


Fig. 11.14 Formation of immature vas deferens with or without a protuberance in the presumptive penis-forming area behind the right tentacle of female *Thais clavigera* exposed to tributyltin (TBT) in a flow-through exposure experiment (Horiguchi et al. 2012a). *p* protuberance as an initial stage of penis formation, *rt* base of the right tentacle, *vd* immature vas deferens. Invagination of epidermal tissue recognised as an immature vas deferens (*vd*), without any protuberance, in the presumptive penis-forming area behind the right tentacle (*rt*) of a female, after 5 days of exposure (A). Invagination of epidermal tissue recognised as an immature vas deferens (*arrow*) behind the protuberance as an initial stage of penis formation (*p*) in the presumptive penis-forming area behind the right tentacle of a female after 7 days of exposure (B). Bars (A) 200 μ m; (B) 100 μ m

specimens had both a penis and vas deferens. However, regarding these two imposex-exhibiting females, a vas deferens was only observed beneath the penis, and no vas deferens was observed close to the vaginal opening of the capsule gland, which is different from the characteristics of vas deferens formation observed in females of a wild *Thais clavigera* population in Hiraiso. One had a vas deferens that opened at the tip and base of the penis, and the other had a vas deferens that was a blind duct. After 24 days of TBT exposure, the five selected female specimens consisted of three imposex-exhibiting females and two apparently normal females. One female had an immature vas deferens as well as a protuberance in the presumptive penis-forming area behind the right tentacle. The vas deferens observed was not close to the vaginal opening of the capsule gland, but it was beneath the penis-like protuberance behind the right tentacle. No vas deferens (i.e., invagination of the epidermal tissue) was observed in the other female specimens after 24 days of TBT exposure. In no control female specimen was the development of a vas deferens observed (Horiguchi et al. 2012a).

Based on the findings from histological observations of specimens from a wild *T. clavigera* population and laboratory flow-through exposure experiments, Horiguchi et al. (2012a) concluded that the invagination of the epidermal tissue in the presumptive penis-forming area behind the right tentacle leading to the formation of the vas deferens would follow on, or occur at almost the same time as, formation of the protuberance in the presumptive penis-forming area of female *T. clavigera*. Rarely, invagination of the epidermal tissue for vas deferens formation may precede the formation of the protuberance in the presumptive penis-forming area. However, invagination of the epidermal tissue close to the vaginal opening of the capsule gland would subsequently occur, leading to the formation of the vas

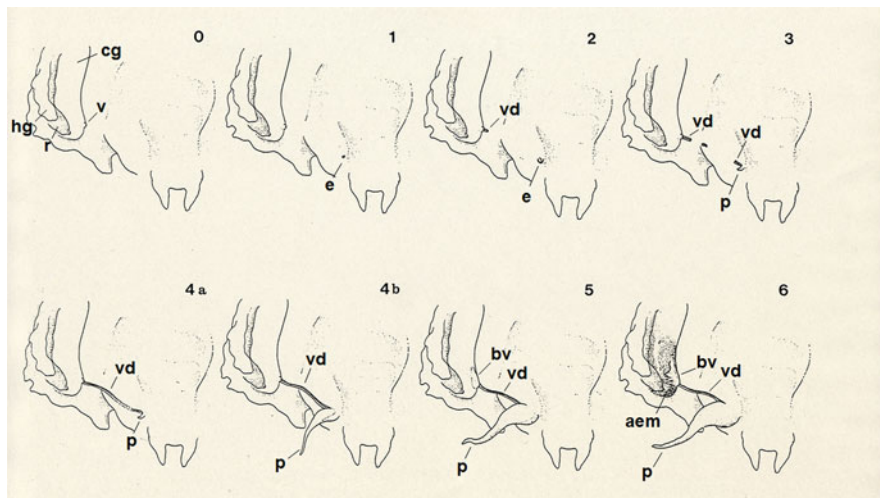


Fig. 11.15 Vas deferens sequence (VDS) index for *Thais clavigera* (Horiguchi et al. 2012a). *aem* aborted egg mass, *bv* blocked vulva, *cg* capsule gland, *e* elliptical protuberance, *hg* hypobranchial gland, *p* penis, *r* rectum, *v* vulva, *vd* vas deferens. VDS 0: Neither penis nor vas deferens is observed (a normal female). VDS 1: A protuberance is observed in the presumptive penis-forming area behind the right tentacle. Invagination of the epidermal tissue in the presumptive penis-forming area is observed, but no invagination of the epidermal tissue is observed close to the vaginal opening (i.e., vulva) of the capsule gland. VDS 2: A protuberance is clearly observed and recognised as an ellipse or an oval in the presumptive penis-forming area behind the right tentacle. Invagination of the epidermal tissue is observed close to the vaginal opening (i.e., vulva) of the capsule gland as well as in the presumptive penis-forming area. VDS 3: The protuberance is apparently/morphologically found to be a tiny penis. Invagination of the epidermal tissue occurs at several locations between the vaginal opening (i.e., vulva) of the capsule gland and the penis, leading to the formation of the vas deferens. The invaginated epidermal tissues extend from several locations and connect to each other to form the duct of the vas deferens. VDS 4: The vas deferens is completed as a duct, and subsequently, the penis grows. VDS 5: The proliferation of the epidermal tissue surrounding the vas deferens covers and blocks the vaginal opening (i.e., vulva) of the capsule gland, resulting in sterility. No aborted egg capsule mass is observed in the capsule gland. VDS 6: In addition to the symptoms seen for VDS 5, an aborted egg capsule mass, darkened and compressed, is observed in the capsule gland

deferens. The number of locations of the epidermal tissue where invagination occurs is not likely fixed, and it may sometimes occur at several locations between the vaginal opening of the capsule gland and the penis; then, the vas deferens beneath the penis would extend toward the tip of the penis. The penis may be differentiated and formed by unidentified aggregated cells in the epidermal tissue (Horiguchi et al. 2012a).

Thus, based on the findings already mentioned, the VDS index for *Thais clavigera* was proposed as follows (Fig. 11.15). VDS 0: Neither the penis nor the vas deferens is observed even in histological preparation; therefore, it is recognised as a normal female. VDS 1: A protuberance is observed in the presumptive penis-forming area behind the right tentacle, and an invagination of the epidermal tissue in the

presumptive penis-forming area could also be observed if a histological examination is conducted, but no invagination of the epidermal tissue is observed close to the vaginal opening of the capsule gland. VDS 2: A protuberance is clearly observed and recognised as an ellipse or an oval in the presumptive penis-forming area behind the right tentacle, and an invagination of the epidermal tissue in the presumptive penis-forming area is also observed if a histological examination is conducted. An invagination of the epidermal tissue is also observed close to the vaginal opening of the capsule gland. VDS 3: The protuberance is apparently/morphologically found to be a tiny penis, and the invagination of the epidermal tissue at several locations between the vaginal opening of the capsule gland and the penis, leading to the formation of the vas deferens. The invaginated epidermal tissues extend from several locations and connect to each other to form the duct of the vas deferens. VDS 4: The vas deferens is completed as a duct, and subsequently, the penis grows. VDS 5: The proliferation of the epidermal tissue surrounding the vas deferens covers and blocks the vaginal opening of the capsule gland; therefore, the release of egg capsules is obstructed. This female is considered to be sterile, but no aborted egg capsule mass is observed in the capsule gland. VDS 6: In addition to the symptoms seen for VDS 5, an aborted egg capsule mass, darkened and compressed, is observed in the capsule gland (Fig. 11.15) (Horiguchi et al. 2012a). Thus, the VDS index for *Thais clavigera* is a little different from that for *Nucella lapillus*, as defined by Gibbs et al. (1987).

As referred to in Chap. 9, the hypothesis that the activation of RXR (Nishikawa et al. 2004) is the mechanism by which TBT and TPhT induce imposex in gastropods seems to be the most likely of the six proposed hypotheses. Interaction between the organotins (i.e., TBT or TPhT) and RXR may occur in the presumptive penis-forming area behind the right tentacle or in the head ganglia, which is the central nervous system of gastropods, soon after exposure to TBT or TPhT, leading to an accumulation of TBT or TPhT in tissues (Horiguchi et al. 2012b). Specific genes and protein expressions could be involved, although the details remain unknown. The downstream physiological pathways may include the processes of differentiation, proliferation, and morphogenesis of the male genitalia (i.e., penis and vas deferens) in both male and imposex-exhibiting female gastropods.

We should also remember that both penis and vas deferens were already observed in males and imposex-exhibiting females from wild populations even at an estimated age of several months, just after settlement, in *T. clavigera* (Fig. 11.16) (Horiguchi et al., unpublished data). This finding is rather different from the age of *Babylonia japonica* completing the development of a vas deferens and penis, as described earlier in this chapter (Table 11.1). In male *B. japonica*, the onset of development of genital organs such as the vas deferens and penis seems to differ from that in imposex-exhibiting *N. lapillus* and *T. clavigera* females. First, it seems to take from 20 to 24 months for male *B. japonica* to develop a complete genital tract (i.e., testicular duct, vas deferens, and penis) and mature testis. On the other hand, the order of formation, with vas deferens formation preceding penis formation in male *B. japonica*, is similar to that in imposex-exhibiting female *N. lapillus*, although it differs from that in imposex-exhibiting female *T. clavigera*, wherein development of the vas deferens does not precede penis development.

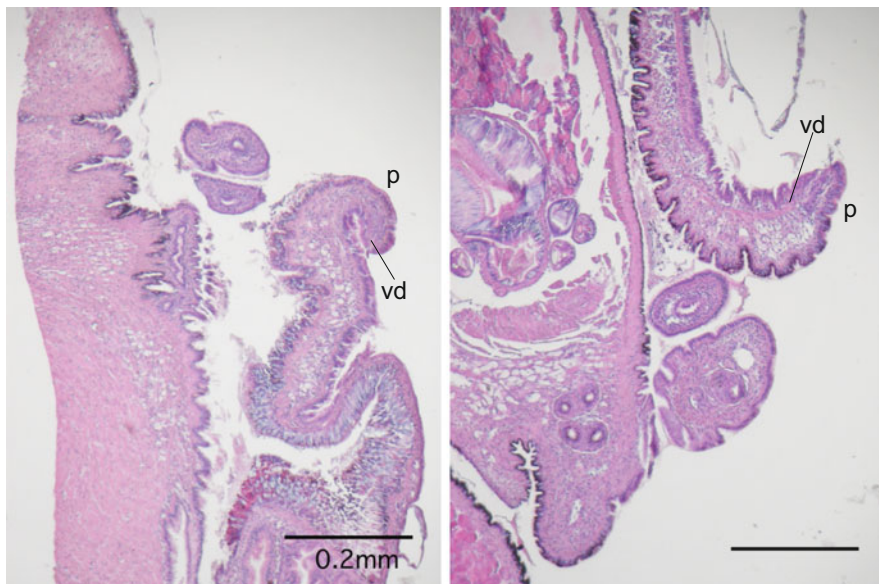


Fig. 11.16 Tiny penis and immature vas deferens observed in wild juvenile male and imposex-exhibiting female *Thais clavigera* at an estimated age of several months, just after settlement (Horiguchi et al., unpublished data). *Left*: male. *Right*: imposex-exhibiting female (shell height approximately 6–7 mm), collected at Jogashima, Japan, on January 10, 2004. *p* penis, *vd* vas deferens. Bar 0.2 mm

Stroben et al. (1995) described a general scheme of imposex development in prosobranch gastropods and illustrated various patterns for the process of development of the vas deferens and penis in imposex-exhibiting females. This scheme suggests that there are various, slightly different, developmental patterns of the vas deferens and penis among prosobranch gastropod species exhibiting imposex. The early process of development of the vas deferens, however, was similar in male *B. japonica* and imposex-exhibiting female *T. clavigera*, in both of which it occurred as an epidermal invagination. These results suggest that the differentiation and development of male-type genitalia in imposex-exhibiting female prosobranch gastropods generally mimic those in male prosobranch gastropods, except for the age at onset and the time to completion. We should also be aware that it does not seem to be strictly fixed or regulated: relatively large variation in the differentiation and development of genitalia could occur among individuals, as well as among species of prosobranch gastropods. It also may imply that, in mollusks, the physiological mechanisms of the differentiation and development of male-type genitalia are less strictly regulated than in vertebrates.

Although the natural ligand of the rock shell RXR and other gastropod RXRs is currently unknown (Horiguchi et al. 2007, 2008, 2010a, b; Urushitani et al. 2011), 9-*cis* retinoic acid (9cRA) is known to be the natural ligand for mammalian RXRs (Heyman et al. 1992; Levin et al. 1992; Mangelsdorf et al. 1992; Mangelsdorf and

Evans 1995). Therefore, the retinoic acids, such as 9cRA, may be important in inducing and promoting the development of the male genitalia in both male and imposex-exhibiting female gastropods (see Chap. 9). Many variations in the development of the vas deferens, the external morphology of the penis, and the modes of blocking the vaginal opening have been observed in imposex-exhibiting female *T. clavigera* (Horiguchi 1993) as well as other gastropod species, such as *Nucella lapillus*, *Ocenebra erinacea*, and *Ilyanassa obsoleta* (Bryan et al. 1986; Gibbs and Bryan 1986; Gibbs et al. 1988, 1990). Although little is known about the physiological functions of retinoic acids in invertebrates, retinoic acids are known to have key roles in embryo patterning and organogenesis in vertebrates (Morris-Kay 1997; Redfern 1997). The ventral/external split of the capsule gland in *T. clavigera* (Horiguchi et al., unpublished data), which is similar to *O. erinacea* (Gibbs et al. 1990), may also be caused by the involvement of RXR. Whether ovarian spermatogenesis (i.e., the sex change by testicular tissue formation in the ovary) in *T. clavigera* and other gastropod species (Gibbs et al. 1988; Horiguchi and Shimizu 1992) is also induced by the involvement of RXR remains unclear.

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