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Authors: Kobayashi, Kazuya, Koyanagi, Ryo, Matsumoto, Midori, Cabrera, Jocelyn Padilla, and Hoshi, Motonori

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Switching from Asexual to Sexual Reproduction in the Planarian *Dugesia ryukyuensis*: Bioassay System and Basic Description of Sexualizing Process

Kazuya Kobayashi*, Ryo Koyanagi, Midori Matsumoto,
Jocelyn Padilla Cabrera and Motonori Hoshi

Department of Life Science, Tokyo Institute of Technology, Nagatsuta, Yokohama 226-8501, Japan

ABSTRACT–An assay system has been established for the sexual induction in the OH strain, an exclusively fissiparous (asexual) strain, of *Dugesia ryukyuensis* by feeding them with sexually matured worms of *Bdellocephala brunnea*, an exclusively oviparous (sexual) species. In this assay system, asexual worms gradually differentiated sexual organs, namely the ovary, testis, genital pore and yolk gland in this order, and eventually mated and laid cocoons filled with fertilized eggs. Although the OH strain worms were believed not to have any sexual organs, a pair of undeveloped ovaries with a few oogonia were detected by an intensive histological search. Along with the progression of sexualization, five distinct stages were histologically recognized: In the first stage, the ovaries became larger enough to be externally apparent; oocytes appeared first at stage 2; the primordial testes emerged at stage 3; a genital pore opened, yolk gland primordia developed and spermatocytes appeared at stage 4; and finally at stage 5 matured spermatozoa and yolk glands were formed. Worms in stages 1 and 2 but not in later stages returned asexual if feeding on *B. brunnea* was interrupted. Furthermore, when the worms at stage 3 onwards were cut posterior to the ovaries, all the tail regenerants developed eventually into fully sexualized worms. Taking these results in account, we have concluded that the process of sexualization has a point-of-no-return between stages 2 and 3. It is likely also that the testes, even the primordia, play an important role in the maintenance and development of sexuality.

INTRODUCTION

It is broadly observed that metazoans occasionally convert the mode of reproduction presumably depending upon the environmental conditions and/or the phase of life cycle. However, the mechanisms underlying the switching from the asexual to the sexual mode of reproduction, and *vice versa*, remain unknown except in the multicellular green flagellate *Volvox*. It is known in *V. carteri* that heat shock elicits production of sexual inducer, a glycoprotein of 30 kDa (Starr and Jaenicke, 1974; Kirk and Kirk, 1986; Mages *et al*., 1988).

Many freshwater planarians (phylum Platyhelminthes, class Turbellaria, order Seriata, suborder Tricladida) comprise races with different modes of reproduction; namely exclusively asexual, seasonally sexual, and exclusively sexual. In the seasonally sexual races, the worms enter the sexual phase in autumn-winter to form hermaphroditic sexual organs and lay cocoons. In spring-summer, their sexual organs degenerate and they reproduce by fissioning until the beginning of the next sexual season (Curtis, 1902; Hyman, 1951). It is possible to sexualize the exclusively asexual worms by changing environmental factors such as temperature in laboratory (Kenk, 1937; Sugino, 1971; Benazzi, 1974) and by transplanting a piece of sexual body into the asexual worms (Kenk, 1941; Okugawa, 1957). They suggested that the testis secreted a sexualizing hormone. On the other hand, it is well known that asexual worms become sexual if they are fed with sexual worms of the same, as well as different, species suggesting that sexual worms contain a sexualizing substance(s) of poor species-specificity (Grasso and Benazzi, 1973; Sakurai, 1981; Teshirogi, 1986). Furthermore, Grasso *et al*. (1975) proposed a hypothesis of a neurosecretory origin of the sexualizing substance(s).

In order to isolate and identify the putative sexualizing substance(s) in the sexual planarians, we have established an assay system for the sexual induction in an exclusively fissiparous (asexual) strain of *Dugesia ryukyuensis*, OH strain, by feeding them with sexually matured worms of *Bdellocephala brunnea*, an exclusively sexual species. By using this assay system, five distinct stages in the sexual induction were histologically recognized. It was also found that the sexual induction in *D. ryukyuensis* had a point-of-no-return after which the worms could spontaneously develop sexual organs without

* Corresponding author: Tel. +81-45-924-5721;
FAX. +81-45-924-5777.

feeding further on *B. brunnea*.

MATERIALS AND METHODS

Animals

An exclusively fissiparous strain (OH strain) of the species *Dugesia ryukyuensis* gifted by Dr. S. Ishida, Hirosaki University, was maintained at 20°C in dechlorinated tap water by feeding with chicken liver. After starvation for two to three weeks, the worms are used as asexual recipients for all experiments. Sexual worms used as the food to sexualize the recipients were wild populations of *B. brunnea* collected in the vicinities of Hirosaki City and Yamagata City, Japan. They were maintained at 5°C in dechlorinated tap water without feeding and used for experiments within a few days.

Five recipients about 10 mm in body length were placed in a plastic dish, ϕ90 mm and fed daily on 10 mg (wet weight) of minced planarians, either *B. brunnea* (experimental) or *D. ryukyuensis* OH strain (negative control) for a period specified for each experiment.

Histology

Recipients were fixed with 4% formalin in PBS for histological examination of the degree of sexualization. Specimens were dehydrated through ethanol series, cleared in xylene and embedded in Paraplast Plus™ (Sherwood Medical, St. Louis, MO) and transverse sections of 4 μm thick were cut. Sections were stained with Mayer's hematoxylin and eosin.

RESULTS

Assay system

We planned to use exclusively asexual worms as the recipient, and exclusively sexual ones as the source of the putative sexualizing substance. Other important issues to be concerned on the animals were the supply (abundance, constancy, ease and readiness), easy culture in laboratory, resistance to operation like ablation, and adequate body size. Taking all these points into account, we chose the OH strain

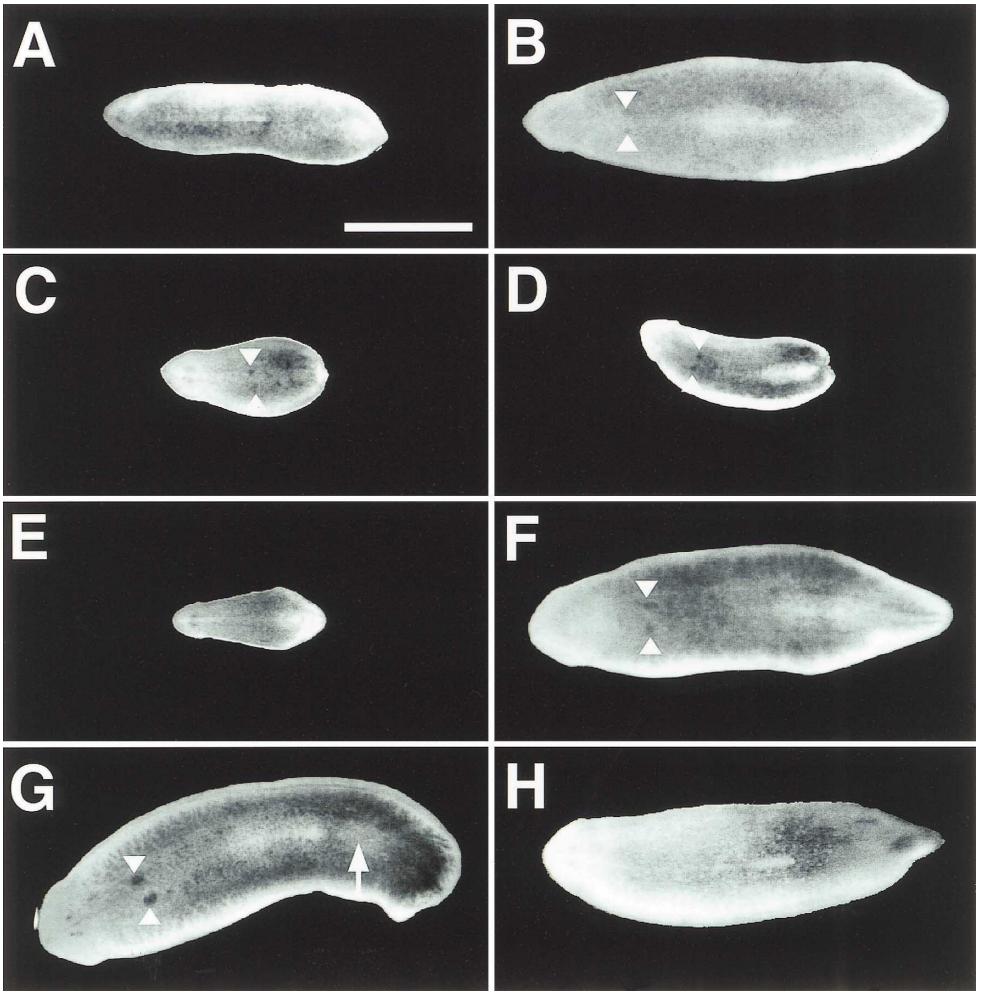

Fig. 1. Anatomical changes during the sexual induction. (A) Asexual worm of *Dugesia ryukyuensis* OH strain. (B) Sexualized worm with a pair of ovaries. (C) Head regenerant without the pharynx from sexualized worm with a pair of ovaries. (D) Head regenerant with the pharynx from sexualized worm with a pair of ovaries. (E) Head regenerants without the pharynx after degeneration of ovaries. (F) Head regenerants with pharynx maintaining the pair of ovaries. (G) Sexualized worm with the ovaries and a genital pore. (H) Control worm after the feeding experiment. All images are ventral view. The ovaries and genital pore are indicated by arrowheads and an arrow, respectively. Fig. A–H are the same magnification (scale bar, 5 mm) and arranged to the anterior on the left.

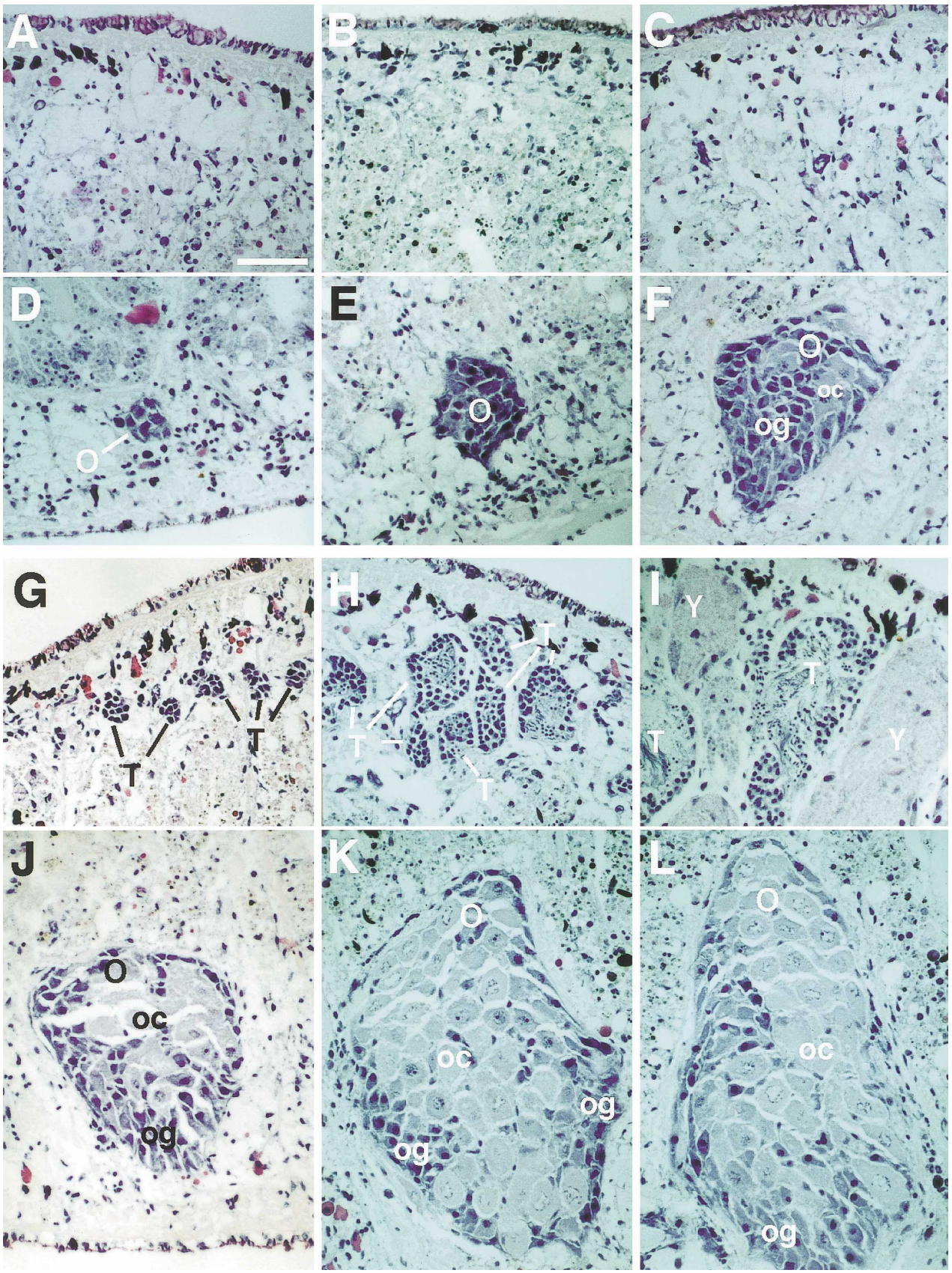

Fig. 2. Development of gonads throughout stages 1 to 5 are shown. Testes: control (A), stage 1 (B), stage 2 (C), stage 3 (G), stage 4 (H) and stage 5 (I). Ovaries: control (D), stage 1 (E), stage 2 (F), stage 3 (J), stage 4 (K) and stage 5 (L). In the image (I), yolk glands are also shown. O, ovary; og, oogonia; oc, oocytes; T, testis; Y, yolk gland. Scale bar, 100 μm.

of *D. ryukyuensis* as the recipient because spontaneous sexual induction has never observed in this strain. As the source of the putative sexualizing substance, we used the adult worms of wild populations of *B. brunnea*, in which only the sexual reproduction is known in the field. Since preliminary experiments showed that the worms of OH strain were sexualized when they were fed with *B. brunnea*, we tried to make the assay period as short as possible. After intensive and extensive survey for the appropriate conditions of the bioassay, such as the population density, feeding procedure, temperature and others (Kobayashi *et al.*, unpublished data), we established a simple, reliable and relatively quick assay system as summarized in MATERIALS AND METHODS.

Anatomical changes along with sexual induction

Fig. 1 summarizes externally observed anatomical changes during sexual induction. No sexual organs were externally recognized in the worms of *D. ryukyuensis* OH strain before or after feeding on the worms of the same strain or chicken liver, they never appeared sexual organs (Fig. 1A and H). However, they were sexualized upon feeding on sexually matured worms of *B. brunnea*, an exclusively sexual species (Fig. 1B–G). First, a pair of ovaries appeared behind the head after about three days of treatment (Fig. 1B). Then, after the treatment for two weeks, the copulatory apparatus became visible as a white speck at the postpharyngeal region, and finally the genital pore opened on the ventral side mostly within 2–3 weeks (Fig. 1G). When worms with a pair of developing ovaries underwent fissioning at the pre- or post-pharyngeal level, they formed head regenerants without or with the pharynx respectively (Fig. 1C and D). In almost all regenerants without pharynx, the ovaries degenerated until they regenerated a functional pharynx to feed on *B. brunnea* (Fig. 1E). However, all the worms without fissioning and regenerants with the pharynx kept their ovaries growing gradually (Fig. 1F) and afterwards opened a genital pore (Fig. 1G). The worms with well-developed ovaries and the genital pore did not undergo fissioning any more, and their sexual organs never degenerated even if feeding on *B. brunnea* was quitted. Eventually, they laid several cocoons filled with fertilized eggs. Almost all juveniles from the cocoons developed into sexual worms.

Histological changes along with sexual induction

Histological studies of recipients were carried out along with the progression of the sexual induction (Figs. 2, 3, 4). Although it was believed that the OH strain worms did not have any sexual organs, a pair of undeveloped ovaries (ovarian primordia) with a few oogonia were revealed by an intensive histological search in the control worms (Figs. 2D, 5A). After feeding the worms with *B. brunnea*, the ovarian primordia grew into the size to be externally visible as mentioned in the previous section (Fig. 2E). After about 7 days of feeding on *B. brunnea*, the oocytes became detectable (Fig. 2F). A pair of ovaries kept developing and the number and size of oocytes increased (Fig. 2J). After 4 weeks of treatment, fully matured oocytes were formed in the well-developed ovaries (Fig. 2K, L). At about Week 3 of treatment, several primordial testes emerged in the dorsal side (Figs. 2G, 3A) and then the testes kept increasing in number and size. About Week 4, spermatocytes appeared and about Week 5 (Figs. 2H, 3B), matured spermatozoa became visible (Figs. 2I, 3C). When the worms developed a genital pore, primordia of yolk glands were observed at ventral region behind the ovaries (Fig. 4A).

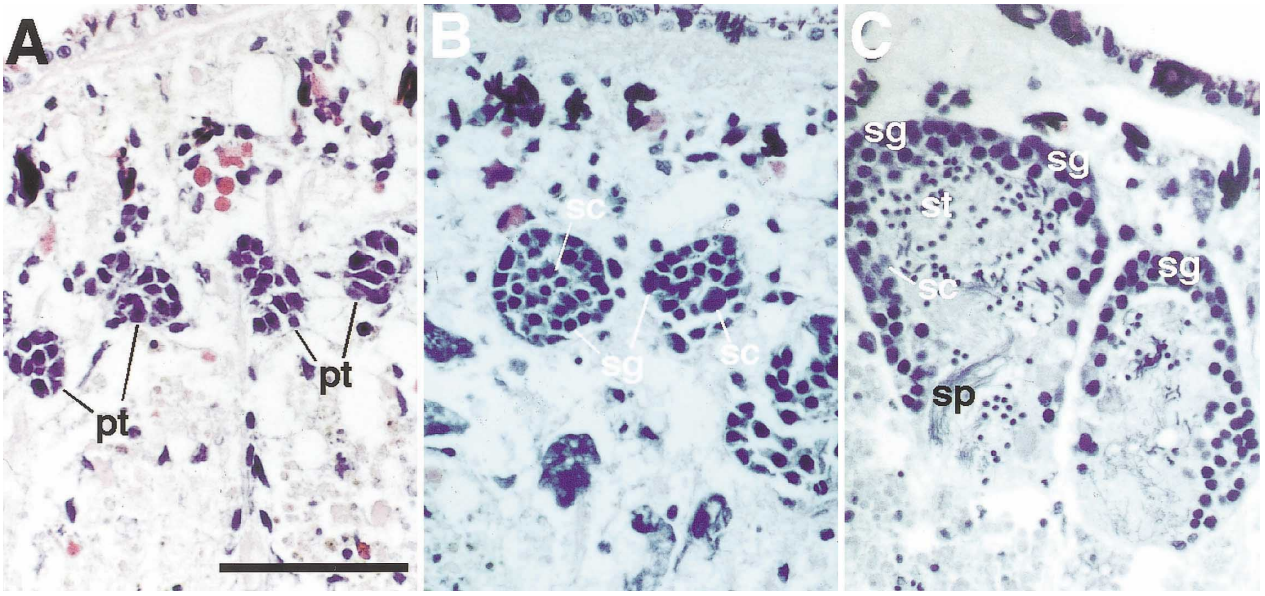

Fig. 3. Developing testes at high magnification. (A) Primordia of testes with the appearance of a nest of neoblast-like spermatogonia in stage 3 worm. (B) Developed tests containing several spermatocytes in stage 4 worms. (C) Well-developed testes contained clustered spermatozoa in stage 5 worms. pt, primordium of testis; sg, spermatogonia; sc, spermatocyte; st, spermatides; sp, spermatozoa. Scale bar, 100 μm.

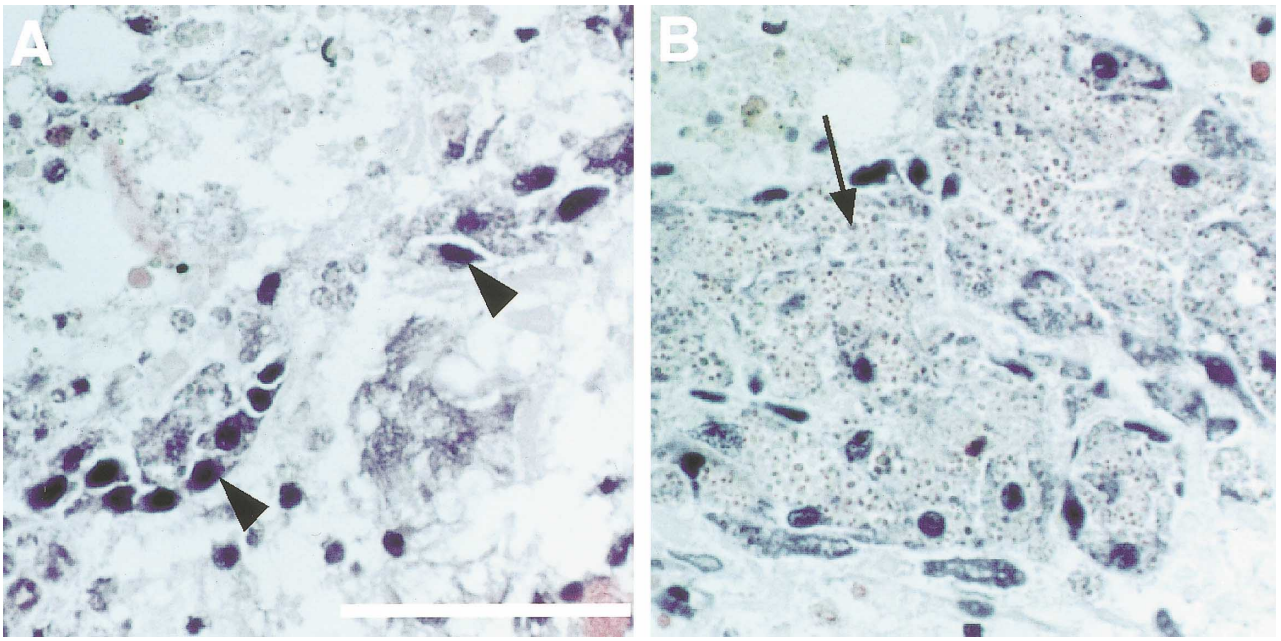

Fig. 4. Development of yolk glands. (A) Primordia of yolk glands (arrowheads) in stage 4 worms. (B) Well-developed yolk gland containing many yolk globules (arrow) in stage 5 worms. Scale bar, 100 μm.

Later, when the testes developed completely with many spermatozoa, the yolk glands had many yolk globules (Figs. 2I, 4B). Based upon morphological changes, we divided the process of sexual induction into five stages as shown in Table 1. Briefly, in stage 1, the ovaries became larger enough to be externally apparent, yet no oocytes nor other sexual organs were detectable. In stage 2, oocytes appeared in the ovaries but other sexual organs remained undetectable. In stage 3, the primordial testes emerged, and in stage 4, a genital pore became externally apparent, yolk gland primordia developed and spermatocytes appeared in the testes. In stage 5, matured yolk glands were formed and many matured spermatozoa were detectable in the testes.

It should be noted that the morphogenesis of the sexual organs during sexual induction proceeds quite similar to the process in the regeneration after surgical ablation (Teshirogi, 1962). Morphology and position of the sexual organs formed were not different from those in the sexual strains of *D. ryukyuensis* (Fig. 5B).

Stability of acquired sexuality

After fed on *B. brunnea* for various durations as indicated in Fig. 6A, they were starved for 2 weeks to examine the dependency of later sexual development upon external sexualizing substance. When worms before opening of a genital pore, namely at stages 1–3, were starved, some of them degener-

Table 1. Five distinctive stages in the sexual induction of *Dugesia ryukyuensis*

	Anatomy[1]		Histology[2]						
			Ovaries		Testes			Yolk glands	
	OV	GP	OG	Oc	Sg	Sc	Sp	P	M
Control worms	–	–	+	–	–	–	–	–	–
Sexualized worms									
Stage 1	+	–	+	–	–	–	–	–	–
Stage 2	+	–	+	+	–	–	–	–	–
Stage 3	+	–	+	+	+	±	–	–	–
Stage 4	+	+	+	+	+	+	–	+	±
Stage 5	+	+	+	+	+	+	+	±	+

Data with 15 control worms and 72 experimental worms picked up randomly are collectively expressed.
[1] OV, a pair of ovaries; GP, genital pore; +, observed; –, not observed.
[2] Og, oogonia; Oc, oocytes; Sg, spermatogonia; Sc, spermatocytes; Sp, spermatozoa; P, primordia of yolk glands; M, matured cells of yolk glands; +, many; ±, some; –, not observed.

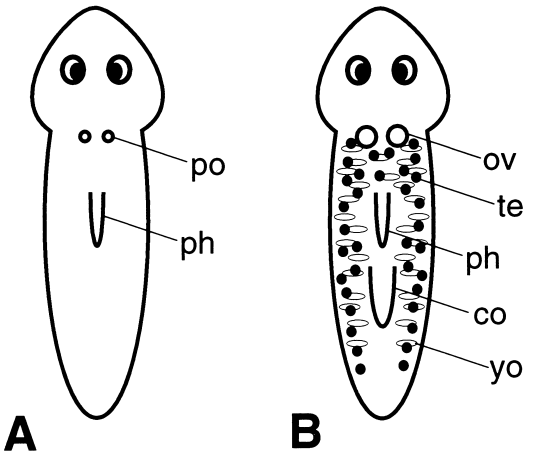

Fig. 5. Illustration of *D. ryukyuensis* OH strain before and after the sexualization. (A) Asexual worm and (B) fully sexualized worm. co; copulatory apparatus; ov, ovary; ph, pharynx; po, primordial ovary; te, testis; yo, yolk gland.

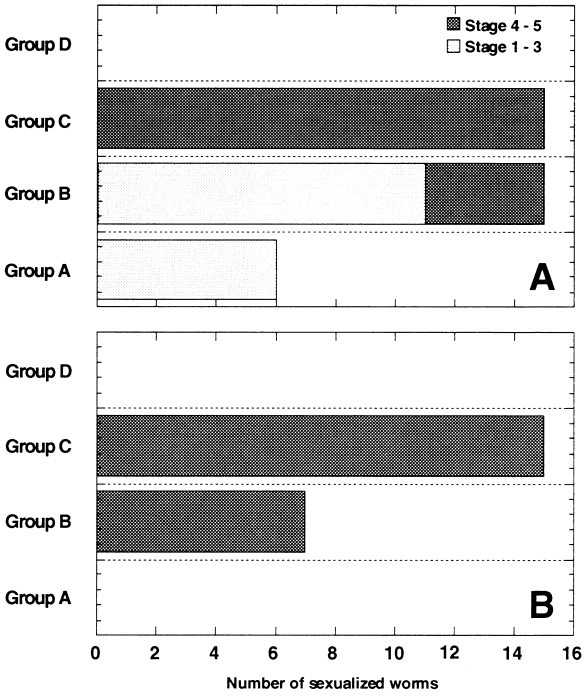

Fig. 6. Effects of abbreviated feeding on *B. brunnea*. Groups A, B and C were fed with sexually matured worms of *B. brunnea* for 1, 3 and 6 weeks, respectively; Group D was fed with asexual worms of *D. ryukyuensis* OH strain for 6 weeks. Each group consists of 15 worms. After the feeding schedule was terminated, the worms were starved for two weeks. The degree of sexualization was scored before (A) and after (B) the starvation.

ated the partially developed ovaries and returned asexual, whereas some others kept developing ovaries and differentiated the genital pore. Those with a genital pore, namely at stage 4 onwards, kept developing the sexual organs even after the starvation (Fig. 6B). Therefore worms became irreversibly sexual somewhere inbetween stages 1–3.

For further examination of the stability of acquired sexuality, the regeneration tests in the sexualizing worms were carried out (Kobayashi *et al.*, in press). The worms with a pair of developing ovaries and those with a pair of well-developed ovaries and a genital pore were cut at their prepharyngeal level. Head fragments were immediately fixed to examine the degree of sexualization histologically. On the other hand, tail fragments were separately placed in a Petri dish of 35 mm in diameter and allowed to regenerate without feeding for a week and then fed on chicken liver once a week for four months to examine their reproductive modes.

Table 2 summarizes the results of regeneration tests. Tail regenerants from the control worms never differentiated functional sexual organs. Those from the sexualizing worms developed sexual organs depending upon the sexualization stage when the worms were cut. Tail regenerants from the stages 1 and 2 worms never developed functional ovaries and returned to asexual. Those regenerants from the stage 3 worms re-

Table 2. Regeneration tests of sexualized worms

	No. of worms tested	Sexual organs in the[1] sexualized worms				Sexual organs in the tail[2] regenerants			
		OV	TE	GP	YG	OV	TE	GP	YG
Stage 0*	15	–	–	–	–	–	–	–	–
Stage 1	9	+	–	–	–	–	–	–	–
Stage 2	11	+	–	–	–	–	–	–	–
Stage 3	14	+	+	–	–	R	Dev	Dif	Dif
Stage 4	11	+	+	+	–	R	Dev	Dev	Dif
Stage 5	15	+	+	+	+	R	M	M	M

* Control worms were expressed as stage 0.

[1] OV, a pair of ovaries; TE, testes; GP, genital pore; YG, matured yolk glands; +, observed; –, not observed.

[2] R, well-developed ovaries were regenerated; M, sexual organs were mainteined during regeneration; Dev, sexual organs after regeneration developed further than those before surgical ablation; Dif, sexual organs were differentiated newly; –, not observed.

generated ovaries and differentiated copulatory apparatus and yolk glands. The primordia of testes observed in stage 3 worms also developed into functional testes. Those regenerants from the stage 4 worms regenerated ovaries and developed functional testes and yolk glands. The copulatory apparatus never degenerated during the course of regeneration. Those regenerants from the stage 5 worms regenerated ovaries and maintained other well-developed sexual organs.

DISCUSSION

Assay system for the sexual induction

In this study, we have established an assay system for the sexual induction in an exclusively fissiparous strain (OH strain) of *D. ryukyuensis* by feeding with sexually matured worms of *B. brunnea*, an exclusively sexual species. Under the feeding schedule used, asexual worms differentiated a pair of ovaries first, then testes, and finally the copulatory apparatus and yolk glands within 3–5 weeks. Since the time required for the sexual induction in this report is much shorter than ever reported (Grasso and Benazzi, 1973; Sakurai, 1981; Teshirogi, 1986), this assay system will help us isolate and identify the putative sexualizing substance. Furthermore, histological data provided us the information to divide the sexual induction into five distinct stages (Table 1), which will give us a useful criterion for the survey of the sexualizing substance.

It is quite interesting to note that the control worms have the primordia of ovaries. Since all the asexual worms of *D. ryukyuensis* have the primordia of ovaries, there is a possibility that other sexual organs also have already been prepared in asexual worms as the primordia. This possibility will be explored by electron microscopy and other techniques.

The point-of-no-return in the sexual induction

Grasso and Benazzi (1973) reported that some of the sexualized worms of *D. gonocephala*, which developed only ovaries, stopped the sexualization process and returned to asexual by fissioning. Our data explained this phenomenon as follows. Head regenerants without the pharynx from the worms with a pair of developing ovaries can not feed on *B. brunnea* simply because they do not have the pharynx, while those with the pharynx can continue feeding on them. In other words, the ovaries under development in the head regenerants without the pharynx degenerate because the supply of the sexualizing substance was interrupted, whereas it is not the case in the regenerants with the pharynx. Therefore the worms with a pair of developing ovaries must be in a phase prior to the point-of-no-return. On the other hand, all the worms with a pair of well-developed ovaries and a genital pore never degenerated their sexual organs and developed into fully sexual worms even after surgical ablation. These observations clearly indicate that the sexual induction in the planarians has a point-of-no-return. In other words, the worms before the point (reversible phase) may need further supply of an external sexualizing substance(s) from *B. brunnea* to acquire the sexuality in full, while those after the point (irreversible phase) may possess an endogenous sexualizing substance(s) or some substances in its downstream to maintain it.

When dose the point-of-no-return occur during the sexual induction? The answer was simply estimated by interruption of feeding with *B. brunnea*. As shown in Fig. 6, some worms of stages 1–3, gradually degenerated the partially developed ovaries unless they were kept feeding on *B. brunnea*, while others maintained their sexual organs and eventually sexualized completely even if feeding on them was quitted. Thus it is concluded that the point-of-no-return is somewhere between stages 1–3. Regeneration tests provided us further information to estimate the point-of-no-return (Table 2). Tail regenerants from the worms at stages 1–2 never became sexual, whereas those from the worms at stage 3 onwards became fully sexual worms. This result indicates that the point-of-no-return is between stages 2 and 3.

Possible requirement of testes for the maintenance of sexuality

When Kenk (1941) and Okugawa (1957) carried out sexual induction by transplanting a piece of sexual body into the asexual worms, the cephalic region without sexual organs did not have the capacity to sexualize the asexual worms. Furthermore, when sexual worms were cut, the head regions without sexual organs became asexual. On the other hand, any regions with sexual organs became fully sexual. Our preliminary experiments showed that the ovaries and the copulatory apparatus seemed not important for the maintenance of sexual development. Thus, not the cephalic region (brain) but testes and/or yolk glands play an important role to maintain the sexuality.

Since the worms of stage 3, that is after the point-of-no-return, have testes already but not yolk glands yet, it is most likely that testes are essential for the maintenance and development of sexuality. This idea is supported by the fact that sexual worms regenerate sexual ones after surgical ablation, whereas asexual worms regenerate asexual ones (Vannini, 1974). Results of the regeneration test summarized in Table 2 provide us a more important line of evidence for the idea. Even though the stage 3 worms lost the ovaries by surgical ablation, the regenerants did regenerate ovaries and differentiated all other sexual organs spontaneously. Thus it is still more likely that the testes, even the primordia, play an important role in the maintenance and development of sexuality.

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