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Authors: Kobayashi, Kazuya, Maezawa, Takanobu, Nakagawa, Haruka, and Hoshi, Motonori

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Existence of Two Sexual Races in the Planarian Species Switching between Asexual and Sexual Reproduction

Kazuya Kobayashi[1]*, Takanobu Maezawa[1], Haruka Nakagawa[1], and Motonori Hoshi[2]

[1]*Center for Integrated Medical Research, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[2]*The Open University of Japan, 2-11 Wakaba, Mihama-ku, Chiba 261-8586, Japan*

In certain planarian species that are able to switch between asexual and sexual reproduction, determining whether a sexual has the ability to switch to the asexual state is problematic, which renders the definition of sexuals controversial. We experimentally show the existence of two sexual races, acquired and innate, in the planarian *Dugesia ryukyuensis*. Acquired sexuals used in this study were experimentally switched from asexuals. Inbreeding of acquired sexuals produced both innate sexuals and asexuals, but inbreeding of innate sexuals produced innate sexuals only and no asexuals. Acquired sexuals, but not innate sexuals, were forced to become asexuals by ablation and regeneration (asexual induction). This suggests that acquired sexuals somehow retain asexual potential, while innate sexuals do not. We also found that acquired sexuals have the potential to develop hyperplastic and supernumerary ovaries, while innate sexuals do not. In this regard, acquired sexuals were more prolific than innate sexuals. The differences between acquired and innate sexuals will provide a structure for examining the mechanism underlying asexual and sexual reproduction in planarians.

Key words: reproductive strategy, asexual reproduction, sexual reproduction, ovary, *Dugesia ryukyuensis*, planarian

INTRODUCTION

Many organisms can reproduce both sexually and asexually. When environmental factors are favorable, asexual reproduction allows animals to exploit conditions suitable for survival. Asexual organisms may switch to a sexual mode of reproduction; sexual reproduction ensures a mixing of the species gene pool. The variations in offspring produced by sexual reproduction allow some individuals to be better suited for survival and provide a mechanism for selective adaptation to occur. Thus, the reproductive strategy of switching between asexual and sexual modes of reproduction may contribute to an organism's fitness. Some freshwater planarians (Platyhelminthes, Turbellaria, Seriata, and Tricladida) switch between asexual and sexual reproduction (Curtis, 1902; Hyman, 1939; Vowinckel, 1970; Vowinckel and Marsden, 1971a, b). The asexuals reproduce by fission without forming any sexual organs (Fig. 1A), whereas the sexuals are hermaphroditic (Fig. 1B). The sexuals produce cocoons (egg capsules) that are filled with multiple fertilized eggs from the ovaries and yolk cells from the yolk glands (Gremigni and Falleni, 1991, 1992, 1998). In planarian embryonic development, the transient organs, such as the embryonic pharynx and transitory intestines, appear first, after which the embryos ingest yolk cells for development and growth (Le Moigne, 1963, 1966; Sanchez-Alvarado, 2003). Embryos also cannibalize their siblings (Harrath et al., 2009). Therefore, there is a considerable variation in the number and size of juveniles from each cocoon (Jenkins, 1967; Renoldson et al., 1983). Embryos do not develop sexual organs until after hatching (Curtis, 1902; Gremigni, 1974). Juveniles of the oviparous species *Bdellocephala brunnea* gradually develop a pair of ovaries, testes, a copulatory apparatus, and yolk glands, in that order (Sakurai, 1998).

Kenk (1937) and Okugawa and Kawakatsu (1954) proposed that the planarians *Euplanaria tigrina* (present name: *Girardia tigrina*) and *Dugesia gonocephala* (present name: *D. japonica*) have at least two physiological races that differ in their manner of reproduction: an asexual and a sexual race. The asexual race reproduces exclusively by fission and does not express sexuality at all. The sexual race can switch to an asexual state spontaneously, and subsequently to revert spontaneously to a sexual state. According to this definition (Kenk, 1937), sexuals that retain sexual reproduction without undergoing asexual reproduction are also categorized in the sexual race. It is further possible that the sexual race contains both sexuals that retain asexual potential and sexuals that probably cannot switch to the asexual state. Benazzi (1974, 1981) described these races as exfissiparous specimens and exclusively sexual specimens,

* Corresponding author. Tel. : +81-3-5843-6085;
Fax : +81-3-5843-6085;
E-mail: kobayashikyram@a5.keio.jp
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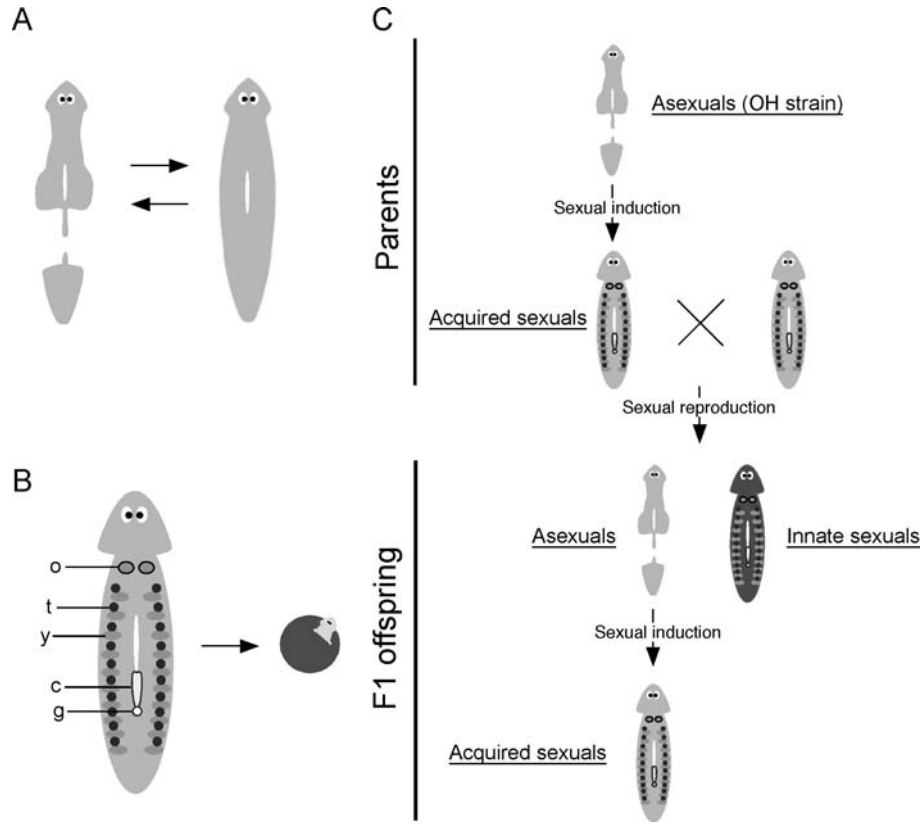


Fig. 1. Illustration of experimental animals. **(A)** Asexual *Dugesia ryukyuensis*. Asexuals undergo transverse fission followed by regeneration without sexual organs. **(B)** Sexual *D. ryukyuensis*. Sexuals are hermaphrodites that produce cocoons filled with several fertilized eggs and many yolk cells. c, copulatory apparatus; g, genital pore; o, ovary; t, testis; y, yolk gland. Usually, a pair of ovaries and a copulatory apparatus (a genital pore) are externally visible in *D. ryukyuensis*. **(C)** Terminology used in this study.

respectively. However, there is no evidence that innate sexuals are not the result of asexuals acquiring sexuality, as the definition of reproductive races was based only on observations in natural habitats and in the laboratory. We think that it is essential to distinguish these two putative races by using rigorous evidence to elucidate the mechanisms underlying sexual and asexual reproduction in planarians.

In certain planarians, asexuals can switch to a sexual state if they are fed sexuals, indicating that sexuals contain a sex-inducing substance or substances (Grasso and Benazzi, 1973; Grasso et al., 1975; Benazzi and Grasso, 1977; Sakurai, 1981; Teshirogi, 1986; Hauser, 1987). Worms of an exclusively asexual strain (the OH strain) of *D. ryukyuensis* can become sexual if fed *B. brunnea* (Kobayashi et al., 1999; Kobayashi and Hoshi, 2002; Kobayashi et al., 2002). We divided the process of sexual induction into five distinct stages on the basis of morphological changes (see Fig. 1 in Kobayashi and Hoshi, 2002, Additional file 1 in Kobayashi and Hoshi, 2011), which coincided with consecutive weeks. For example, stage 1 was in week 1 of the feeding treatment, stage 2 in week 2, etc. In stage 1, the ovaries grew sufficiently large to be externally apparent behind the head, although no oocytes or other sexual organs were detectable. Oocytes appeared in the ovaries, but other sexual organs remained undetectable in stage 2. In stage 3, the primordial testes emerged, and a copulatory apparatus became visible as a white speck in the post-pharyngeal region, and in stage 4, yolk gland primordia developed and spermatocytes appeared in the testes. Finally, in stage 5, the mature yolk glands formed and many mature spermatozoa were detectable in the testes. It should be noted that the morphogenesis of the sexual organs during sexual induction was quite similar to that observed post-embryogenesis (Sakurai, 1998). We found that the test worms in stages 1 and 2 returned to being asexual when feeding on *B. brunnea* was stopped. However, sexual induction has a "point of no return" between stages 2 and 3 (indicating that they will not become asexual after this point), and test worms in stage 3 onward continued developing sexual organs, though feeding on *B. brunnea* was stopped. We concluded that worms with acquired sexuality (acquired sexuals) no longer needed to feed on *B. brunnea* to maintain their sexuality.

The acquired sexuals copulate and produce offspring (Hoshi et al., 2003; Kobayashi et al., 2008). Recently, we showed that the offspring consisted of both asexuals and sexuals (Kobayashi et al., 2009). The asexuals became acquired sexuals after feeding on *B. brunnea*, like the worms of the OH strain did, whereas the sexuals (innate sexuals) became sexually mature without feeding on *B. brunnea* (Fig. 1C) (Hoshi et al., 2003). Externally, innate sexuals were not easily distinguishable from acquired sexuals. Previously, we could not exclude the possibility that the innate sexuals were asexual, like the acquired sexuals. In the present study, we provide a rationale that considers the potential to become asexual by ablation-regeneration (asexual induction) and ovarian morphology as a means of distinguishing between acquired and innate sexuals. Based on the results, we have revisited the definition of reproductive races in planarians.

MATERIALS AND METHODS

Animals

An exclusively asexual strain, the OH strain of the planarian *D. ryukyuensis* (Kawakatsu et al., 1976; Kawakatsu et al., 1995), was donated by Dr. S. Ishida, Hirosaki University, Japan. Acquired sexuals were obtained by feeding asexuals *B. brunnea* (Ijima and Kaburaki, 1916), as described previously (Kobayashi et al., 1999). Worms were maintained at 20°C in dechlorinated tapwater, and were fed chicken liver once a week.

Maintenance of juveniles

A group of approximately 30 acquired sexuals derived from the OH worms was maintained at 20°C in dechlorinated tap water by

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being fed chicken liver once a week. Under these conditions, they laid approximately 100 cocoons within approximately two weeks. We selected 100 juveniles to observe and record the expression of their reproductive traits. The juveniles were separated into groups of 10. Each group was separately maintained in approximately 50 mL of dechlorinated tap water and fed chicken liver once a week. A binocular microscope was used to observe the specimens once a week for 20 weeks to determine whether the juveniles developed sexual organs, and specifically, a copulatory apparatus. When the juveniles underwent fission, the tail fragments were removed to maintain a constant population density. The number of first fissions was also recorded.

Ablation-regeneration tests

For the ablation-regeneration tests, worms were cut as described previously (Kobayashi et al., 2002). Fragments were transferred separately to a plastic dish (3 cm in diameter) containing dechlorinated tap water, allowed to regenerate at 20°C, and fed chicken liver once a week. During the ablation-regeneration tests, external observations were performed weekly to determine whether regenerated worms became sexual or asexual.

Whole-mount in situ hybridization

Worms were relaxed in cold 2% (vol/vol) HCl in 5/8 Holtfreter's solution (Betchaku, 1970) for 5 min, fixed in 4% paraformaldehyde (PFA), placed in a 5% methanol solution at 4°C for 3 h, and bleached in hydrogen peroxide/methanol (1:4 [vol/vol]) under fluorescent light at room temperature (RT) for approximately 12 h. They were hydrated in a series of decreasing concentrations of methanol, treated with proteinase K (20 μg/mL; Nacalai Tesque, Kyoto, Japan) in PBTw (0.1% Tween 20 in phosphate-buffered saline) at 37°C for 12 min for asexuals or 20 min for sexuals, and post-fixed in 4% PFA solution at RT for 30 min. The specimens were then washed three times in PBTw for 10 min. The specimens were incubated in a prehybridization solution (50% formamide, 5 × SSC, 1 mg/mL yeast tRNA [Roche Applied Science, Mannheim, Germany], 100 μg/mL heparin, 0.1% Tween 20) at 56°C for 1 h and hybridized with a digoxigenin (DIG)-labeled antisense RNA probe (approximately 50 ng/mL) in prehybridization solution supplemented with 10% dextran sulfate at 56°C for 16 h. A probe for *Dr-nanos* was synthesized from the clone Dr_sW_028_K12 containing the *nanos* gene (Ishizuka et al., 2007). The probe was hydrolyzed by adding an equal volume of AB solution (80 mM $NaHCO_3$, 120 mM Na_2CO_3, 10 mM DTT [dithiothreitol]) and incubated at 60°C for 60 min. The hydrolysis reaction was quenched with an equal volume of NB solution (300 mM sodium acetate, 1% acetic acid, 10 mM DTT), and the probe was purified by ethanol precipitation. After hybridization, the specimens were washed six times in a solution of 50% formamide, 5 × SSC, and 0.1% Tween 20 at 56°C for 30 min; once in a solution of 20% formamide, 2 × SSC, and 0.1% Tween 20 at 56°C for 30 min; and two times in a solution of 2% formamide, 0.2 × SSC, and 0.1% Tween 20 at 56°C for 30 min. Then, they were washed three times in MABTw (pH 7.5, 100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20) at RT for 10 min. They were incubated in a blocking solution (MABTw containing 1% blocking reagent [Roche Applied Science, Mannheim, Germany] and 10% sheep serum) at RT for 1 h and then incubated in the blocking solution with an alkaline phosphatase-conjugated Fab fragment against DIG (Roche Applied Science, Mannheim, Germany) at RT for 3 h. After the specimens were washed in MABTw four times for 15 min and four times for 30 min, they were incubated with AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, topped up to volume with 10% polyvinyl alcohol solution) for 15 min. Specimens were subjected to color development by incubation at RT in AP buffer containing nitro blue tetrazolium (170 μg/mL, Roche Applied Science, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl phosphate (175 μg/mL, Roche Applied Science, Mannheim, Germany).

Histology

Worms were fixed in 10% formalin in phosphate-buffered saline. The fixed specimens were dehydrated through an ethanol series, cleared in xylene, and embedded in Paraplast Plus embedding medium (Sigma-Aldrich Co., St. Louis, MO, USA). The embedded specimens were cut into 4-μm thick sections and stained with hematoxylin and eosin.

Statistical analysis

Statistical analysis was performed using the chi-square test to determine the occurrence of asexuals and sexuals in the ablation-regeneration tests. The induction of supernumerary ovary pairs and the cocoon production between acquired and innate sexuals were evaluated using Student's *t*-test.

RESULTS

Development of juveniles produced by inbreeding of acquired sexuals from the OH strain

We previously showed that inbreeding of acquired sexuals in the OH strain produces both asexuals and innate sexuals at a ratio of 2:1 with a significance level of 5% (Kobayashi et al., 2009). No reproductive traits were expressed on the newly hatched worms. On reaching a suitable body size, some juveniles and their offspring ("fissioned" worms) underwent fission and growth without developing sexual organs. We classified these offspring as asexuals, and the total number of the asexuals was thus expressed as the cumulative number of first fissions (Fig. 2A). As there was considerable variation in the number and size of juveniles from each cocoon, the appearance of the asexuals (noted by observation of first fission) gradually increased after a 5-week period as more of the juveniles reached a suitable size. External observations revealed that the remaining juveniles, who were innate sexuals, developed a copulatory apparatus without undergoing fission after a 4-week period (Fig. 2B, C). Approximately one week after the appearance of a copulatory apparatus, a genital pore opened on the ventral side of the juvenile animals (Fig. 2D). In sexual induction of the OH strain, the appearance of a copulatory apparatus and genital pore meant that the worms were at stages 3 and 4, respectively (see Introduction). Even in juveniles with a genital pore, a pair of ovaries was not externally visible, although at stage 1 in sexual induction, this became sufficiently large to be apparent behind the head (for the position of ovaries, see the pair of black arrowheads in Fig. 3B). Histological research revealed that the juveniles with a genital pore not only had mature ovaries with oocytes, but also developing testes and primordia of yolk glands (Supplementary Fig. S1). This degree of sexual development was identical to that in stage 4 of sexual induction. In fact, other than the difference in ovarian size, the morphogenesis of sexual organs was nearly identical in the sexual development of juveniles and sexual induction of asexuals. When the offspring (juveniles) became sexually mature, a pair of ovaries became externally apparent (Fig. 3D). These juveniles could be therefore identified as innate sexuals.

Asexual induction in acquired and innate sexuals

We showed that acquired sexuals can be forced to become asexuals by ablation and regeneration (asexual induction). Head (H) fragments regenerated to become

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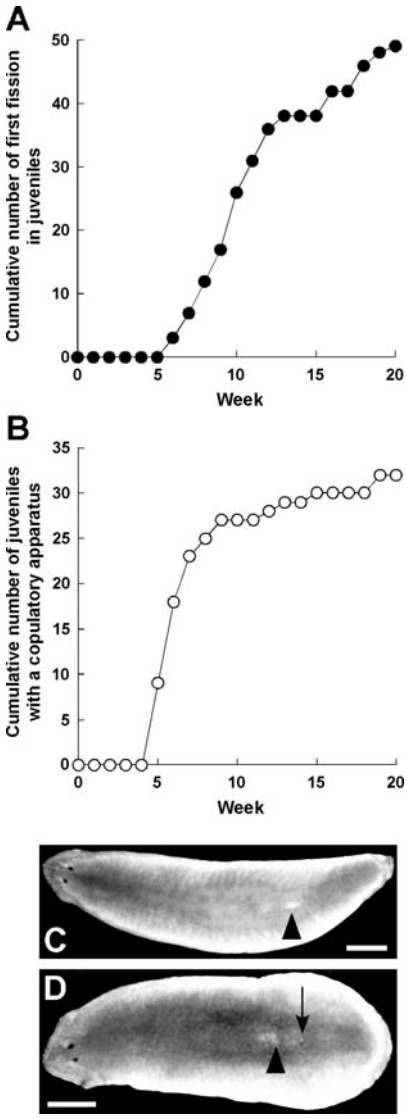


Fig. 2. Expression of reproductive traits in juveniles obtained by inbreeding of acquired sexuals. About 100 juveniles were used in this observation. **(A)** Cumulative number of first fission in the juveniles. Forty-nine juveniles began to undergo fission. Later, they asexually reproduced by fission without developing sexual organs. Therefore, the cumulative number of first fission was regarded as the number of asexuals. **(B)** Cumulative number of the juveniles developing a copulatory apparatus. Thirty-two juveniles externally developed a copulatory apparatus without undergoing fission. Later, they became sexually mature and were regarded as innate sexuals. Ventral view of a juvenile with a copulatory apparatus **(C)** and a juvenile with a genital pore **(D)**. Arrowheads represent a copulatory apparatus. An arrow represents a genital pore. The image is arranged with the anterior side at the left. Scale bar: 1 mm. Nineteen juveniles died before the expression of these reproductive traits. They seemed to not grow under the laboratory conditions.

asexual, and middle (M) and tail (T) fragments regenerated to become sexual (for the position of the fragments, see Fig. 2 in Kobayashi et al., 2002 and Fig. 5 in Kobayashi and Hoshi, 2011). This raised the question of whether fragments from innate sexuals regenerate to become asexual or sexual. In the present study, we conducted ablation-regeneration tests on innate sexuals. In acquired sexuals, the M and T

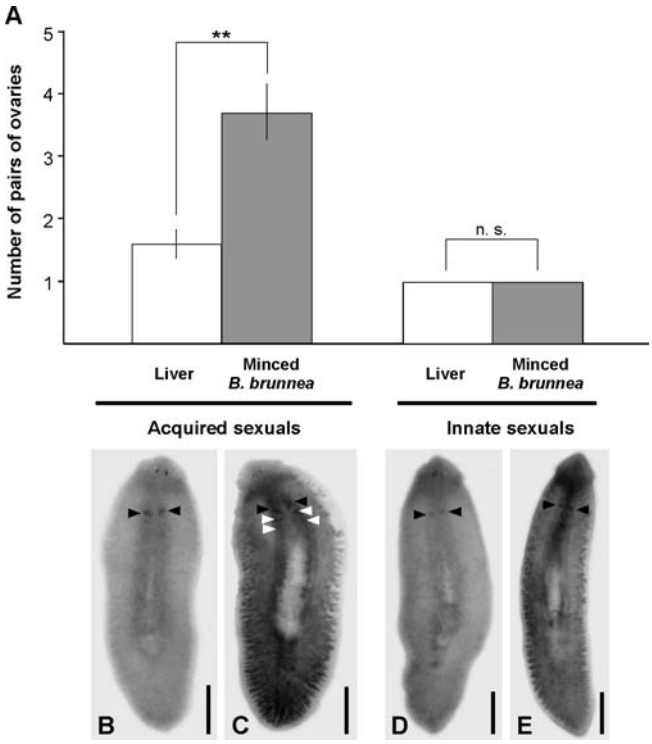


Fig. 3. Induction of supernumerary ovary pairs in acquired and innate sexuals. **(A)** Significantly more supernumerary ovary pairs were induced in acquired sexuals fed *Bdellocephala brunnea* for two weeks than in those fed chicken liver for two weeks (Student's *t*-test: $n_{B.\ brunnea} = 10$, $n_{Liver} = 5$; $P = 0.0077$). In contrast, no supernumerary ovary pairs were induced in innate sexuals, even if they were fed *B. brunnea*. Error bars represent the standard error. **(B–E)** Morphological examination in acquired and innate sexuals. Ventral view of an acquired sexual fed chicken liver **(B)** and *B. brunnea* **(C)** and an innate sexual fed chicken liver **(D)** and *B. brunnea* **(E)**. Arrowheads represent a main ovary (black) and a supernumerary ovary (white). The image is arranged with the anterior side at the top. Scale bar: 1 mm.

Table 1. Reproductive mode of regenerated worms from acquired and innate sexuals.

Origins of regenerated worms	Fates of regenerated worms			
	Asexual	Sexual	Dead	Unknown
Acquired sexuals				
Head region	46	0	3	0
Middle region	0	49	0	0
Tail region	0	49	0	0
Innate sexuals				
Head region	0	50	14	3*
Middle region	0	67	0	0
Tail region	0	67	0	0

A statistically significant difference was found in the occurrence of asexuals and sexuals in worms regenerated from acquired and innate sexuals (chi-square test: $P = 7.7E\text{-}21$, degrees of freedom = 1). *Regenerated worms did not develop any sexual organs and did not undergo fission. They were maintained for at least four years.

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fragments regenerated to become sexual, whereas the H fragments regenerated to become asexual. However, in innate sexuals, even the H fragments regenerated to become sexual (Table 1).

Ovarian morphology and fecundity in acquired and innate sexuals

In the previous section, innate sexuals were distinguished from acquired sexuals by ablation-regeneration tests. To investigate other differences between acquired and innate sexuals, we focused on ovarian morphology. We observed differences in ovarian size during the sexual development of juveniles and sexual induction of asexuals. However, we could not rule out the possibility that the difference in ovarian size could also have been caused by differences in diet. Acquired sexuals developed ovaries after eating large amounts of *B. brunnea*. Innate sexuals in contrast did not require this food source to develop reproductive organs.

Asexuals became fully sexual, and induction of several supernumerary ovary pairs occurred along the ventral nerve cord, reaching the pharyngeal level, when they were fed *B. brunnea* (Sakurai, 1981; Kobayashi and Hoshi, 2011). We hypothesized that the supernumerary ovaries are a unique morphological feature of acquired sexuals. The acquired sexuals and the innate sexuals were fed *B. brunnea* daily for two weeks. Supernumerary ovaries developed in acquired, but not innate sexuals (Fig. 3). Furthermore, histological research revealed that the main ovaries (black arrowheads in Fig. 3B–E) were noticeably larger in acquired than in innate sexuals (Fig. 4A–B). The increased size of the ovaries in the acquired sexuals was attributed to an increase in the number of female germ cells. The innate sexuals did not develop such ovaries even after being fed *B. brunnea* for two weeks (Fig. 4C). No histological differences were observed in the testes and yolk glands (Fig. 4D–F).

It has been reported that a gene homolog of *nanos*, which is required for germ cell differentiation and maintenance (Forbes and Lehman, 1991; Kobayashi et al., 1996; Hayashi et al., 2004), is a marker of putative germline stem cells in planarians (Sato et al., 2006; Wang et al., 2007; Handberg-Thorsager and Saló, 2007). Recently, in *D. ryukyuensis*, cells positive for the *nanos* homolog (*Dr-nanos*) were identified in the presumed region of supernumerary ovaries both in acquired sexuals and in asexuals (the OH stain) (Nakagawa et al., in press, GenBank accession number AB650592). In this study, we performed whole-mount in situ hybridization of *Dr-nanos* in the innate sexuals (Fig. 5). Even in acquired sexuals with a pair of externally apparent ovaries (Fig. 5A), cells positive for *Dr-nanos* were observed in the presumed region of the supernumerary ovaries (red arrowheads in Fig. 5B). In innate sexuals, however, they were not detectable in this reason (Fig. 5D). Interestingly, *Dr-nanos* was expressed in the brain (yellow arrowheads in Fig. 5D) in innate sexuals only.

These differences in ovarian morphology suggested a difference in fecundity in the two groups of worms. Sexuals lay cocoons filled with several fertilized eggs and many yolk cells. We examined the production of cocoons in acquired

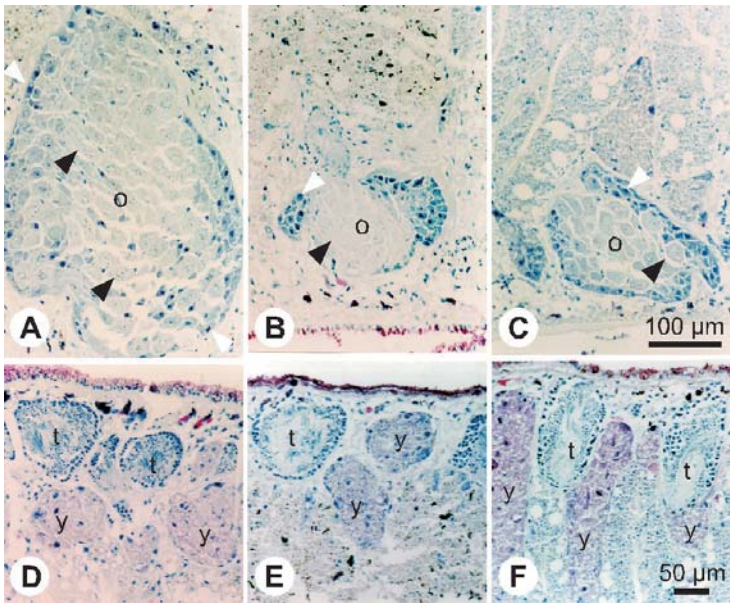


Fig. 4. Ovarian morphology in acquired and innate sexuals. **(A–C)** Main ovary and **(D–F)** testes and yolk glands. **(A, D)** Acquired sexuals. **(B, E)** Innate sexuals. **(C, F)** Innate sexuals fed *Bdellocephala brunnea* daily for two weeks. Figures **(A–C)** and **(D–F)** are at the same magnification. o, ovary; t, testis; y, yolk gland. Black and white arrowheads in **(A–C)** represent an oocyte and an oogonium, respectively.

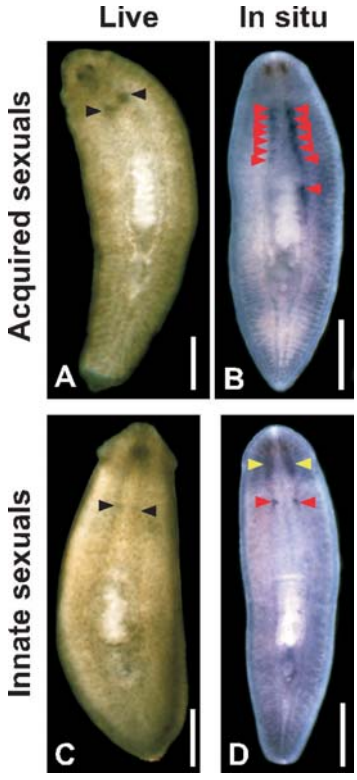


Fig. 5. *Dr-nanos* expression on ventral side of acquired and innate sexuals. **(A–B)** Acquired sexuals and **(C–D)** innate sexuals. **(A, C)** Live images. **(B, D)** Detection of *Dr-nanos* expression by whole-mount in situ hybridization. Arrowheads represent an externally visible main ovary (black), *Dr-nanos* expression in an ovary (red), and *Dr-nanos* expression in the brain (yellow). The image is arranged with the anterior side at the top. Scale bar: 1 mm.

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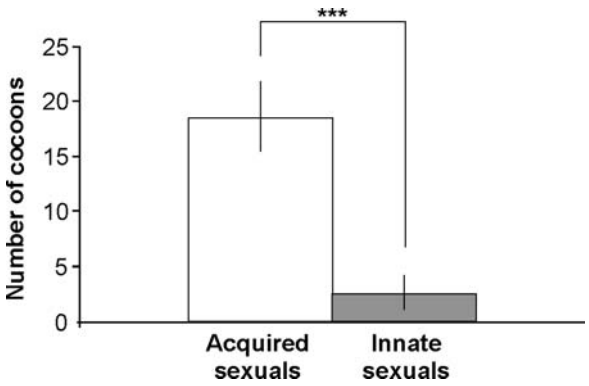


Fig. 6. Cocoon production in acquired and innate sexuals. Acquired sexuals and innate sexuals were maintained separately for three months in the laboratory condition (see Materials and Methods). Acquired sexuals produced significantly more cocoons than innate sexuals (Student's *t*-test: $n_{Acquired} = 8$, $n_{Innate} = 8$; $P = 0.00052$). Error bars represent the standard error.

Table 2. Reproductive mode of F2 offspring from the OH strain.

Parents	Sexual	Asexual
Acquired sexuals from Fis-1*	4	32
Acquired sexuals from 9-1*	4	20
Innate sexuals	27	0

*Both strains were asexuals, which were obtained by the inbreeding of acquired sexuals from the OH strain. They became acquired sexuals by feeding on *Bdellocephala brunnea*. Approximately 30 acquired sexuals from each of the two strains were allowed to mate, and approximately 50 innate sexuals were allowed to mate.

and innate sexuals. As expected, the number of cocoons produced by acquired sexuals was approximately 7-fold higher than that produced by the innate sexuals (Fig. 6). We showed that inbreeding of acquired sexuals of the OH strain produced F1 offspring comprising both asexuals and innate sexuals at a ratio of 2:1 (Kobayashi et al., 2009). We examined inbreeding of innate sexuals and found that the resultant offspring were all innate sexuals (Table 2). In contrast, the inbreeding of acquired sexuals from F1 asexuals produced offspring comprising both innate sexuals and asexuals, as with the OH strain. It should be noted that inbreeding of acquired sexuals from asexuals produced many more asexuals than that of acquired sexuals from the OH strain (Table 2).

DISCUSSION

We found that inbreeding of acquired sexuals from the OH strain produced both asexuals and innate sexuals at a ratio of 2:1, with a significance level of 5% (Fig. 1C) (Kobayashi et al., 2009). This result prompted us to consider why innate sexuals, in which the external organs were indistinguishable from those of acquired sexuals, emerged without having fed *B. brunnea*. Previously, we concluded that sex-inducing substances contained in sexuals induce the production of the sex-inducing substances in otherwise asexual worms. Once the worms acquire sexuality, they begin to produce the sex-inducing substance (Kobayashi et al., 2002). The acquired sexuals were cut into three pieces (the ablation-regeneration test), which were allowed to regenerate. Head (H) fragments regenerated to become asexual, whereas middle (M) and tail (T) fragments regenerated to become sexual (Kobayashi et al., 2002; additional file 3 in Kobayashi and Hoshi, 2011). Recently, we showed that the activity of the sex-inducing substance was recognized in M and T fragments, but not in H fragments (Kobayashi and Hoshi, 2011). These results strongly suggest that the putative sex-inducing substance is involved in the maintenance of acquired sexuality. In other words, the sexuality in acquired sexuals may be maintained by a sex-inducing substance, which "stimulates" its synthesis. The production of such a sex-inducing substance in acquired sexuals (asexuals) may be regulated epigenetically. This indicates that acquired sexuals retain asexual potential. First, we showed that the ablation-regeneration tests in this study could distinguish between acquired and innate sexuals. Head fragments in acquired sexuals regenerated to become asexual. However, those of innate sexuals regenerated to become sexual, apparently despite the lack of a sufficient amount of sex-inducing substance (Table 1). The sexuality in innate sexuals may be expressed by a sex-inducing substance, which is constitutively produced, and the production of this putative substance in innate sexuals may be determined genetically. This is consistent with the sexual development of juveniles that do not feed on *B. brunnea*, and suggests that innate sexuals cannot switch to the asexual state.

Next, we examined the potential to induce supernumerary ovaries in acquired and innate sexuals by feeding on *B. brunnea*. We showed that, in acquired sexuals, the development of supernumerary ovary pairs was induced by feeding on *B. brunnea* (Fig. 3). Innate sexuals did not develop supernumerary ovary pairs, even when fed *B. brunnea* (Fig. 3A, E). We focused on *Dr-nanos*, a marker gene of putative germline stem cells in *D. ryukyuensis*. Even in asexuals (the OH strain), *Dr-nanos*-positive cells were identified in both the presumed region of the main ovaries and in that of the supernumerary ovaries (Nakagawa et al., in press). However, in innate sexuals, *Dr-nanos*-positive cells were not detected in the presumed region of the supernumerary ovaries (Fig. 5). Innate sexuals cannot induce supernumerary ovaries because they cannot form the necessary germline stem cells. Additionally, we found that *Dr-nanos* was strongly expressed in the brain in the innate sexuals (yellow arrowheads in Fig. 5D). In *Drosophila*, *nanos* is essential for dendrite morphogenesis in peripheral neurons (Ye et al., 2004). In mice, there are three homologs of *nanos*. *nanos 1* is expressed in the central nervous system (Haraguchi et al., 2003). Handberg-Thorsager and Saló (2007) showed that *Smed-nanos* is expressed in germ cells as well as eye precursor cells in the planarian *Schmidtea mediterranea*. Thus, *Dr-nanos* expression in the brain may be associated with the differentiation of neurons. However, we cannot currently address why *Dr-nanos* expression in the brain was seen only in innate, and not in acquired sexuals.

We also found that the main ovaries were larger in the acquired sexuals (black arrowheads in Fig. 3B–C) than in the innate sexuals (black arrowheads in Fig. 3D–E), even when the innate sexuals were fed *B. brunnea* (Fig. 4). This enlarged morphology resembles the hyperplastic ovaries observed in ex-fissiparous specimens of European and

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North African *Dugesia* species (Grasso and Benazzi, 1973; Gremigni and Banchetti, 1972a, b; Charni et al., 2004). Acquired sexuals were more prolific than innate sexuals (Fig. 6). They laid numerous cocoons filled with several fertilized eggs and many yolk cells. Benazzi (1981) reported that fecundity was higher in ex-fissiparous specimens of *D. sanchezi* than in exclusively sexual specimens. The hyperplastic ovary (and/or supernumerary ovaries), observed in the acquired sexuals only, may contribute to this fecundity. It may be that planarians become more prolific when they switch from an asexual to a sexual mode of reproduction, because the switch generally occurs when individual survival is threatened. The fitness of acquired sexuals seems to be higher than that of innate sexuals; however, innate sexuals undoubtedly transmit sexuality to their offspring (Table 2). Innate sexuals may contribute to the reproductive success of the species by ensuring sexual reproduction (a guarantee of sex).

For the offspring obtained by inbreeding of the acquired sexuals from the OH strain, the ratio of asexuals to innate sexuals (2:1) resembles a Mendelian ratio (Kobayashi et al., 2009). In this study, inbreeding of innate sexuals produced only innate sexuals, while inbreeding of acquired sexuals from asexuals produced many more asexuals than inbreeding of acquired sexuals from the OH strain (Table 2). Probably, the determination of whether the worm is asexual or innate sexual is genetically controlled. However, we cannot currently address it adequately because the OH strain of *D. ryukyuensis* is triploid, and inbreeding of acquired sexuals in the OH strain produced both diploid and triploid offspring at a ratio of 1:2 or 1:3 with a significance level of 5% (see Table 1 in Kobayashi et al., 2008). In nature, *D. ryukyuensis* (n = 7) exhibits the following three ploidy types: diploidy (2x = 14), triploidy (3x = 21) and mixoploidy (2x + 3x) (Oki et al., 1981; Tamura et al., 1991). There are at least four biotypes: diploid asexuals, triploid asexuals, diploid sexuals, and triploid sexuals (Tamura et al., 1995). In inbreeding of the acquired sexuals from the OH strain, we observed diploid asexuals, triploid asexuals, diploid innate sexuals, and triploid innate sexuals. Most of the diploid offspring were innate sexuals, while two-thirds of the triploid offspring were asexuals (Table 3 in Kobayashi et al., 2009). If the putative genetic factor determining whether being either asexual or innate sexual was a dominant Mendelian trait, we presumably would not have observed this conflicting result in the diploid and triploid offspring. This suggests that the determination of whether the worm is asexual or innate sexual is not necessarily inherited in only a Mendelian fashion. In addition, we must consider the competition of embryos for survival in the cocoons (Le Moigne, 1963, 1966; Sanchez-Alvarado, 2003; Harrath et al., 2009) if we perform a classic genetic analysis of triclads. Further studies are needed to resolve this problem.

In planarians that are able to switch between asexual and sexual reproduction, the definition of sexuals is controversial. In this study, we experimentally showed differences between acquired and innate sexuals in terms of asexual induction and ovarian morphology. Here, we propose two races for the sexual race described by Kenk (1937) and Okugawa and Kawakatsu (1954): an acquired sexual race (probably ex-fissiparous specimens by Benazzi [1974, 1981]) and an innate sexual race (probably exclusively sexual specimens by Benazzi [1974, 1981]). The criteria established in this study will provide a structure for examining the mechanism underlying sexual and asexual reproduction in planarians.

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