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Signification of the Sexualizing Substance Produced by the Sexualized Planarians

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ABSTRACT—Asexual worms of an exclusively fissiparous strain (the OH strain) of the planarian *Dugesia ryukyuensis* keep developing hermaphroditic reproductive organs and eventually undergo sexual reproduction instead of asexual reproduction, namely fission, if they are fed with sexually mature worms of an exclusively oviparous planarian, *Bdellocephala brunnea*, suggesting that the sexually mature worms has a sexualizing substance(s). The fully sexualized worms no longer need the feeding on sexual worms to maintain the sexuality. Here, we demonstrate that the sexualized worms produce enough of their own sexualizing substance similar to that contained in *B. brunnea*. In case of surgical ablation of the sexualized worms, the fragments with sexual organs regenerate to become sexual, while those without sexual organs, namely head fragments, regenerate to return to the asexual state. The asexual regenerants from the sexualized worms are also fully sexualized by being fed with *B. brunnea*. Additionally, it was reported that head region in sexually mature worms lacks the putative sexualizing substance necessary for complete sexualization (Sakurai, 1981). These results suggest that the fragments without sexual organ lack enough of an amount of the putative sexualizing substance and the sexuality is maintained by the sexualizing substance contained in the sexualized worms.

Key words: planaria, asexual-sexual switch, sexualization, sexualizing substance, maintenance of sexuality

INTRODUCTION

Some planarians, especially in the genus *Dugesia*, may reproduce asexually or sexually or by a combination of both methods from population to population within a species (Jenkins, 1967). Worms propagating by asexual reproduction, namely the asexual race, do not have sexual organs and undergo transverse fission. Those reproducing by sexual reproduction, namely the sexual race, have hermaphroditic sexual organs and copulate and then produce cocoons containing several fertilized eggs. Those alternating between asexual and sexual reproduction, namely the 'physiological race' (Jenkins, 1967), develop sexual organs during the colder months of the year, but, when the breeding season is over, the sexual organs degenerate and fission occurs during the warmer months. Although many ecological investigations have revealed the existence of the physiological race (Curtis, 1902; Hyman, 1939), it seems to be difficult to realize the alternation in laboratory conditions. In general, many metazoans can convert the reproductive mode depending upon the environmental conditions and/or the phase of life cycle. However, the mechanisms underlying the switching from the asexual to the sexual reproduction, and *vice versa* remain unknown. In planarians, it is well known that even the asexual race, which reproduces exclusively by fission according to observation over a period of several years and is not sexualized by any environmental factors, becomes sexual, if its worms are fed with sexual worms of the same, as well as different, species (Grasso and Benazzi, 1973; Benazzi and Grasso, 1977; Sakurai, 1981). This result suggests that sexual worms contain a sexualizing substance(s) of poor species-specificity. Although the experimental sexualization in planarians is of benefit to examine the mechanisms underlying the switching from the asexual to the sexual reproduction, the reversed phenomenon, namely 'asexualization' is studied even less.

We established an assay system for the sexualization

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in the OH strain, an exclusively fissiparous strain, of *Dugesia ryukyuensis* by feeding them with sexually mature worms of *Bdellocephala brunnea*, an exclusively oviparous species (Kobayashi *et al*., 1999). We divided the process of sexualization into five distinct stages by morphological changes (Fig. 1) and found that sexualization has a point-of-no-return between stages 2 and 3. The worms at stages 1 and 2 return to being asexual if feeding on *B. brunnea* is stopped. On the contrary, the worms at stages 3 onward keep developing sexual organs, even though feeding on *B. brunnea* is stopped. Simultaneously, the worms at stages 3 onward stop fissioning, and eventually never return to an asexual state (Kobayashi and Hoshi, 2002). Thus, the putative sexualizing substance in *B. brunnea* would not need to maintain the sexuality in the worms after the point-of-no-return, though it causes at least the asexual worms to acquire the sexuality. This result suggests that the worms after the point-of-no-return have already started producing enough of their own sexualizing substance similar to that contained in *B. brunnea*. Even if the sexualized worms produce the sexualizing substance, which gives full play to switch from asexual to sexual reproduction in asexual individuals, it remains unknown what part the chemical plays in sexual individuals.

Okugawa (1957) carried out sexualization by transplanting a piece of worm in the sexual race into the asexual worms. The piece with sexual organs had the capacity to sexualize the asexual region, whereas the head region without sexual organs did not. In general, it is well known that 'neoblasts' in planarians seem to be totipotent stem cells (Baguñà *et al*., 1994, Agata and Watanabe, 1999). Baguñà *et al*. (1989) reported that injection of neoblasts from the sexual race of *Dugesia mediterranea* into x-ray irradiated asexual hosts transformed them into sexual worms. This result suggests that the worms in the sexual race express sexuality spontaneously. On the other hand, Sakurai (1981) reported that head pieces lack some of the substances necessary for complete sexualization because asexual worms fed with head pieces of an oviparous species were incompletely sexualized, while those fed with fragments other than the head were fully sexualized. This result suggests that head region does not have enough of the putative sexualizing substance to sexualize the asexual worms. Thus, the failure to sexualize the asexual region in the experiment of Okugawa (1957) may be attributed to a lack of neoblasts in the sexual race in contrast with that in the asexual race, lack of the putative sexualizing substance, or both. If the sexualizing substance were responsible for maintenance of the sexuality, elimination of the sexualizing substance would cause the sexual individuals to return to an asexual state. The hypothesis would be demonstrated by examining the reproductive mode of the regenerants from the head region, because the effect of the sexualizing substance in the head region is mostly excluded by surgical ablation according to the putative localization of the sexualizing substance. At least, the regeneration experiment based on the hypothesis as described above must lead to whether or not the sexualizing substance is associated with the maintenance of sexuality. In the case of an oviparous species like *B. brunnea*

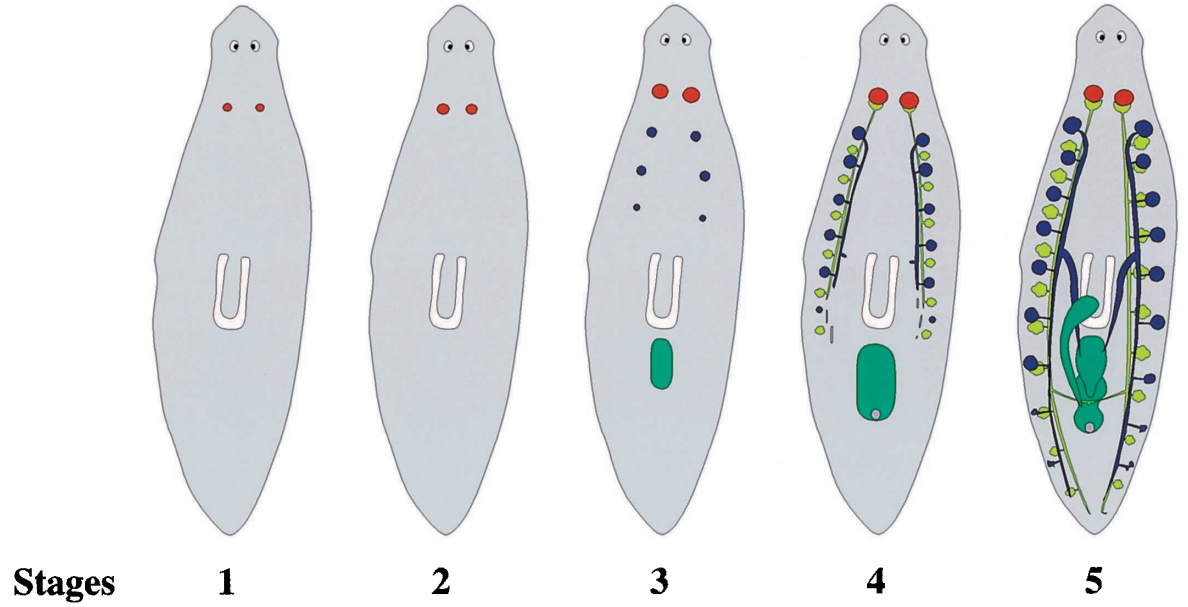


Fig. 1. Illustration of five distinct stages along with the sexualization. The development and topological position of reproductive organs are shown. Red region: ovary; blue region: testis and seminal duct; yellow region: yolk gland and oviduct; green region: a copulatory apparatus with a genital pore. Briefly, at stage 1, the ovaries become larger enough to be externally apparent, yet neither oocytes nor other sexual organs are detectable. At stage 2, oocytes appear in the ovaries. At stage 3, the primordial testes and a copulatory apparatus emerge, and at stage 4, a genital pore in the copulatory apparatus become externally apparent, primordial yolk glands develop and spermatocytes appear in the testes. At stage 5, mature yolk glands are formed and many mature spermatozoa are detectable in the testes.

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and the sexual race, it is difficult to consider that they are asexualized since they do not seem to switch the reproductive mode. Unfortunately, the preliminary experiment by surgical ablation failed because of poor regenerative capacity of the head region in *B. brunnea*. In the case of the fully sexualized worms, there is no evidence whether they are able to switch to asexual individuals like the worms in the physiological race. In this study, we first examined whether or not the sexualized worms of *D. ryukyuensis* have the putative sexualizing substance, and focused on the reproductive mode of the regenerants from the head region in the sexualized worms.

MATERIALS AND METHODS

Animals

An exclusively fissiparous strain, the OH strain of the planarian *Dugesia ryukyuensis* presented by Dr. S. Ishida of Hirosaki University was maintained at 20°C in dechlorinated tap water by being fed with chicken liver. The strain is a clone propagated by fission from a single asexual worm of *D. ryukyuensis* and never shows any evidence for sexuality since 1984. After starvation for two to three weeks, the worms were used as asexual recipients for a feeding experiment. Sexualized worms derived from the OH strain, which were previously sexualized by being fed with the sexually mature worms of *Bdellocephala brunnea* (Kobayashi *et al*., 1999), were maintained under the same conditions as the intact OH strain. The sexually mature worms were used as the food to sexualize the recipients and for a regeneration experiment.

Preparation of the food for sexualization

Sexualized worms (360 worms; total wet weight 4g) derived from the OH strain were homogenized in 120 ml of phosphate-buffered saline (34 mM NaCl, 7 mM KCl, 2.5 mM Na_2HPO_4, 4.5 mM KH_2PO_4, pH 7.4). The homogenate was centrifuged at 100,000 x *g* for 1 hr at 4°C and the supernatant was fractionated by using Sep-Pak Cartridge, tC_{18} Porapak® (Waters™). The fraction eluted with water was mixed with chicken liver homogenate, and then freeze-dried as the food for sexualization. Sexually mature worms of *B. brunnea* (total wet weight 4g), which were collected in the vicinities of Yamagata City, Japan, were also similarly prepared as a positive control. Twenty-five recipients were fed daily on a piece of the food over 4 weeks. The feeding experiment was performed as previously described with some modifications (Kobayashi *et al*., 1999).

Estimation of sexualization

In order to examine the degree of the sexualization, we examined the expression for S21 gene. The S21 gene was isolated by the technique of differential screening between the asexual worms in the OH strain and the worms at stage 3 (Hase *et al*., unpublished data). Along with the progression of the sexualization, the expression of the S21 gene was first observed in a lot of neoblast-like cells located in the ventral parenchymal region at stage 3. Since the expression became to be localized in primordial and mature yolk glands at stages 4 and 5 respectively, the S21 gene seems to be involved in differentiation of the yolk glands. Therefore, we used the S21 gene as a marker for the point-of-no-return in the recipients. The total RNA of the recipients was prepared by a method of guanidium-isothiocyanate/phenol-chloroform (Chomczynski and Sacchi, 1987). To analyze RNAs by northern analysis (Alwine *et al.,* 1997), we separated 7.5 μg of total RNAs on 1% agarose gel containing formaldehyde, transferred them to a positively charged nylon membrane (Pall Biodyne B). Antisense P^{32}-labeled cDNA probes were prepared by a random prime labelling system (Amersham Pharmacia Biotech). Hybridization was carried out at 42°C for 16 hr in the hybridization solution (4x standard sodium citrate [SSC], 50% [v/v] formamide, 0.2% [w/v] sodium dodecylsulfate [SDS], 5x Denhardt's solution and 0.12 mg/ml salmon sperm DNA). Post-hybridization washing was carried out at 50°C for 10 min in a 2x SSC-0.1% SDS. Then, the signals were detected by a BAS 5000 Bio-Image Analyzer (Fuji Photo Film).

Regeneration experiment

As shown in Fig. 2, the sexualized worm of *D. ryukyuensis* was cut into three pieces with a razor. Immediately after surgical ablation, the three fragments were transferred to dechlorinated tap water in a plastic dish with 3 cm in diameter, respectively, and then allowed to regenerate there at 20°C by being fed on chicken liver once a week. External observation was carried out every week. When the regenerants developed sexual organs without fissioning, we regarded them as sexual worms. On the contrary, when they fissioned, we continued to maintain them. We regarded the fissioned offspring as asexual worms, when they fissioned further without developing sexual organs.

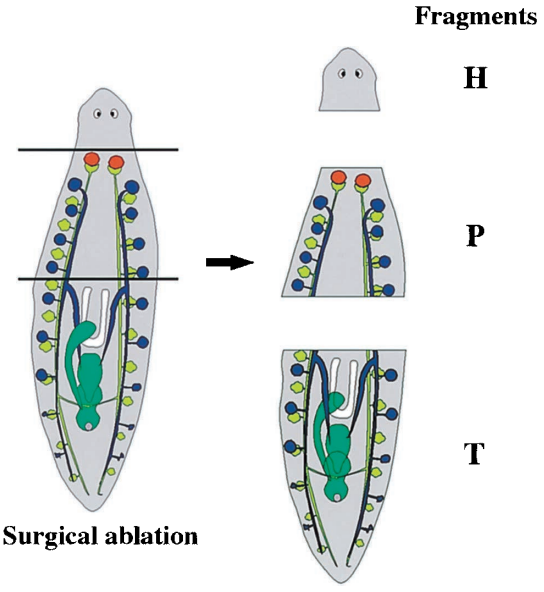


Fig. 2. Scheme of the surgical ablation of the sexualized worms. By the surgical ablation, three different fragments were obtained with respect to the topological position of the sexual organs; H (Head) fragment has no sexual organ; P (Prepharyngeal) fragment has a pair of ovaries, testes and yolk glands; T (Tail) fragment has testes, yolk glands and a copulatory apparatus. Colored regions correspond with the sexual organs described in the figure legend of Fig. 1.

RESULTS

Feeding with sexualized worms derived from the OH strain

External observation showed that some recipients of the OH strain developed a pair of ovaries and a genital pore by the 4 weeks of feeding (Fig. 3A), when fed with the extract of the sexualized worms derived from the OH strain. As shown in Table 1, a pair of developed ovaries appeared in all recipients fed with extract of the sexualized worms, and a genital pore opened in about a half of the recipients. At the same time, the genital pore was not observed in the

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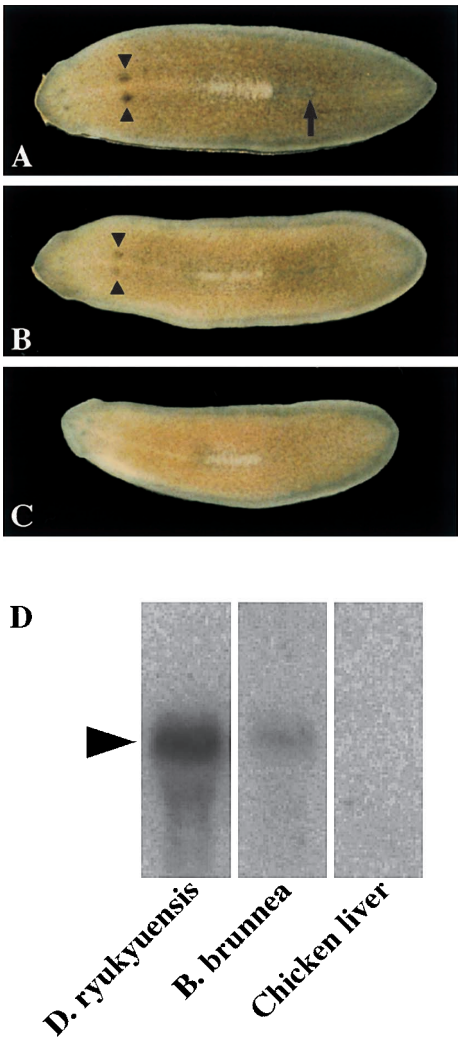


Fig. 3. **(A–C)** Ventral view of recipients at the 4th week of feeding. The recipient fed with the sexualized worms of *D. ryukyuensis* developed a pair of developed ovaries (arrowheads) and a genital pore (an arrow) **(A)**. The recipient fed with *B. brunnea* developed only a pair of developed ovaries (arrowheads) **(B)**. No sexual organs were externally recognized in the recipient after being fed on chicken liver (control) **(C)**. Figs. A–C are the same magnification and arranged to the anterior on the left. **(D)** Northern blot analysis of the S21 gene which is a marker for the point-of-no-return. Ten recipients each were used for total RNA extraction after the feeding experiment. Expression of the S21 gene was detected in the recipients fed with the sexualized worms or *B. brunnea* (an arrowhead). Worms fed with chicken liver, a daily food for planarians, were used as a control.

Table 1. Sexualized appearance of recipients at the 4th week of feeding treatment

Food	Number of recipients that developed a pair of ovaries(%)	Number of recipients that differentiated a genital pore(%)
D. ryukyuensis	25 (100)	13 (52)
B. brunnea	25 (100)	0 (0)
Chicken liver	0 (0)	0 (0)

recipients fed with the extracts of sexually mature worms of *B. brunnea*, though a pair of ovaries appeared in all the recipients (Fig. 3B). No sexual organs were externally recognized in the recipients after being fed on chicken liver which is the daily food to maintain planarians (Fig. 3C).

In order to examine extensively and intensively whether feeding on the sexualized worms fully sexualize recipients or not, we analyzed the expression of a marker gene, the S21 gene (Hase *et al*., unpublished data) for the point-of-no-return in the recipients. The expression of the S21 gene was detected in the recipients fed with the extract of the sexualized worms, as well as that of *B. brunnea* (Fig. 3D).

Regeneration experiment of the sexualized worms derived from the OH strain

We carried out the surgical ablation of them according to putative localization of a sexualizing substance(s) (Sakurai, 1981). By the surgical ablation, three different fragments were obtained with respect to the topological position of the sexual organs (Fig. 2); H (head) fragment has no sexual organ; P (prepharyngeal) fragment has a pair of ovaries, testes and yolk glands; T (tail) fragment has testes, yolk glands and copulatory apparatus. Fragments from each five sexualized worm were allowed to regenerate. As a result of the regeneration (Table 2), all P and T regenerants developed the sexual organs without fission. On the contrary, H regenerants never developed them, and continued to fission. In the first generation of the regeneration, P and T regenerants were sexualized without being fed on sexual specimens. In order to confirm whether or not the sexual regenerants maintain this feature, we continued the regeneration experiment until the third generation of the regeneration was obtained. As shown in Table 3, all P and T regenerants developed sexual organs without fission, while all H regenerants became asexual. At third generation of the regeneration, the fragments with and without the sexual organs also became sexual and asexual respectively (data not shown).

Table 2. Reproductive mode of regenerants from the sexualized worms

Sexualized worms	Regenerants obtained by surgical ablation[a]		
	H	P	T
Ac-2	A	S	S
Ac-4	–[b]	S	S
Ac-5	A	S	S
Ac-7	A	S	S
Ac-8	A	S	S

A: fissioned regenerants without developing sexual organs;
S: regenerants differentiated full sexual organs without fission.
[a] At the time of the surgical ablation, difference of regenerants (fragments) is described in Fig. 2.
[b] The regenerant died after failing to regeneration.

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Table 3. Reproductive mode of regenerants at second generation of regeneration

Sexual worms	Regenerants obtained by surgical ablation		
	H	P	T
P regenerants from the sexualized worms			
Ac-2	A	S	S
Ac-4	A	S	S
Ac-5	A	S	S
Ac-7	A	S	S
Ac-8	A	S	S
T regenerants from the sexualized worms			
Ac-2	A	S	S
Ac-4	A	S	S
Ac-5	A	S	S
Ac-7	A	S	S
Ac-8	A	S	S

For explanation of symbols, see Table 2.

As a result of the regeneration experiment, five fissiparous strains from the H regenerants were obtained (Tables 2 and 3). In order to examine whether asexual worms of these strains were sexualized by the effects of the exogenous sexualizing substance or not, we carried out feeding experiments by using sexually mature worms of *B. brunnea* (Kobayashi *et al*., 1999), and then examined the expression of the S21 gene in the recipients. After 3 weeks of the feeding treatment, all the recipients from each five strain expressed the S21 gene as well as those of OH strain (Fig. 4). Indeed, they were sexualized externally (data not shown).

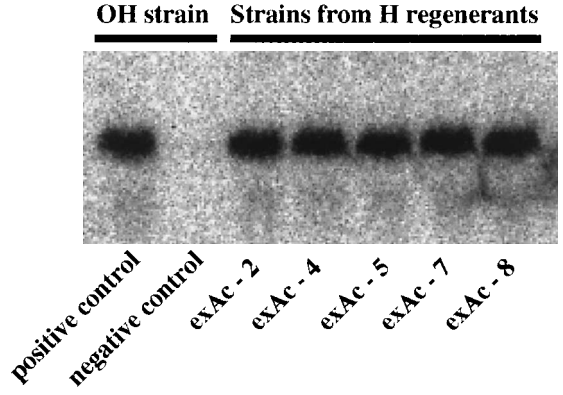


Fig. 4. Northern blot analysis of the S21 gene in fissiparous strains obtained from the sexualized worms. Five fissiparous strains were obtained by surgical ablation of the sexualized worms. Ten worms from each strain were fed with the sexually mature worms of *B. brunnea* for three weeks everyday. After the feeding treatment, all the recipients from each strain were used for total RNA extraction. The recipients of each strain expressed the S21 gene as well as those of OH strain. Worms fed with chicken liver were used as a negative control.

DISCUSSION

Sexualization by feeding on the sexualized worms derived from the OH strain

In nature, the physiological race may alternate between asexual and sexual reproduction seasonally (Curtis, 1902; Hyman, 1939; Jenkins, 1967). Although one of the factors for this alternation seems to be changes of temperature, it is likely that the realization is difficult in a laboratory. On the other hand, it is suggested that sexual worms contain a sexualizing substance(s) of poor species-specificity (Grasso and Benazzi, 1973; Benazzi and Grasso, 1977; Sakurai, 1981). However, the putative sexualizing substance has not been isolated and identified yet, and the relationship between the sexualizing substance and sexuality is still obscure. We established an assay system for the sexualization in the OH strain, an exclusively fissiparous strain, of *Dugesia ryukyuensis* by feeding them with sexually mature worms of *Bdellocephala brunnea*, an exclusively oviparous species (Kobayashi *et al*., 1999). In this assay system, fully sexualized worms no longer need the feeding on sexual worms to maintain the sexuality. Sexuality may be maintained in three different ways: (1) the sexualizing substance contained in *B. brunnea*, directly and irreversibly, sexualizes the worms, (2) that induces the synthesis of a 'true' sexualizing substance(s) that sexualize the worms irreversibly, (3) that triggers the synthesis of a 'true' sexualizing substance(s) that reversibly sexualizes the worms and stimulates its synthesis *per se*. We examined whether or not the worms sexualized by being fed with *B. brunnea* produce the sexualizing substance physiologically.

In the feeding experiment, we observed sexualization by feeding with the sexualized worms derived from the OH strain (Fig. 3 A–C, Table 1). We also observed histologically the developing sexual organs in the recipients after the treatment of feeding (data not shown). In addition to the external observation, the histological examination revealed that, after being fed with the sexualized worms and with *B. brunnea,* the recipients corresponded with worms at stages 4 and 3, respectively (Fig. 1). The degree of sexualization was quite compatible with the expression of the S21 gene, a marker of the point-of-no-return (Fig. 3D). Although the rate of sexualization was a little different between feeding on the sexualized worms and *B. brunnea* (Table 1), the process of that was identical. These results clearly indicate that the feeding of sexualized worms of *D. ryukyuensis* induced the sexuality in the asexual worms. Although the sexualized worms for the feeding experienced to be fed with the sexualizing substance contained in *B. brunnea*, the remainder of the substance within the sexualized worms would not seem to contribute to the sexualization of this experiment. Sexualized worms cut by surgical ablation usually regenerated to become sexual without being fed with *B. brunnea*. After several cycles of the regeneration, the sexual worms have the capacity of sexualization similar to that of the sexualized

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worms (data not shown). If only the sexualizing substance of *B. brunnea* was responsible for sexualization, the feeding of the sexual worms after several cycles of the regeneration would not induce the sexuality in the asexual worms. Thus, this result suggests that the sexualized worms produce enough of an amount of their own sexualizing substance similar to that contained in *B. brunnea*. The differences (quality and/or quantity) of the sexualizing substance contained in the sexualized worms and *B. brunnea* might contribute to the different rates of sexualizing process described above (Table 1). We can conclude that the action of the putative sexualizing substance contained in sexual planarians induces the production of the sexualizing substance in otherwise asexual worms.

Asexualization of the sexualized worms by surgical ablation

In previous section, we showed that the sexualized worms maintaining the sexuality keep producing the endogenous sexualizing substance. What is the relationship between the exogenous and/or endogenous sexualizing substance and the sexuality? We observed in the sexualized worms that head (H) fragments without sexual organs regenerated to become asexual until the third generation of the regeneration experiment, while prepharyngeal (P) and tail (T) fragments with sexual organs returned to sexual state (Tables 2 and 3). Furthermore, some fragments (about a half of the fragments) also became asexual, when the region with sexual organs was cut the same as the H fragments concerning about neoblast number (counted as described by Baguñà *et al*., [1989]) and fragment volume (data not shown). The fact of the asexualization of H fragments indicates that the sexualized worms are committed reversibly by the exogenous and/or endogenous sexualizing substance. As a result, the two possibilities (1) and (2) described above are contradicted. The asexualization in H fragments seems to be caused by a lack of the putative sexualizing substance, because all fissiparous strains from the H regenerants were sexualized by the exogenous sexualizing substance (Fig. 4). This may correspond to the reports of Sakurai (1981) that head region of sexually matured planarians lacks some of the substances necessary for complete sexualization. We can conclude that the sexualized worms keep their sexuality depending upon the endogenous sexualizing substance. As a result, the sexualized worms may return to asexual worms like the worms in the physiological race. However, the worms in the physiological race naturally switch the reproductive mode. Probably, they are able to regulate the production of the sexualizing substance depending upon the environmental conditions. On the other hand, the worms in the sexual race and the oviparous species would spontaneously express the sexuality by the constitutive production of the sexualizing substance; that is to say, they may be not asexualized. This hypothesis supports the result of Baguñà *et al*. (1989) as described in INTRODUCTION. The 'true' action of the sexualizing substance, which was shown in this study, would be a clue to elucidate diversification of the complicated reproductive strategies in planarians as described in INTRODUCTION.

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