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Seasonal Changes in the Sexualization of the Planarian *Dugesia ryukyuensis*

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ABSTRACT—Asexual individuals in a fissiparous clone of the planarian *Dugesia ryukyuensis* develop hermaphroditic sexual organs and eventually undergo sexual reproduction instead of asexual reproduction if they are fed with the adults of *Bdellocephala brunnea*, an oviparous planaria. The experimental sexualization means that the adults of *B. brunnea* contain a putative sexualizing substance(s), which is the first candidate for the chemical(s) responsible for switching from asexual to sexual reproduction in metazoans. In the present study, the feeding experiment over two consecutive years revealed that the experimental sexualization has seasonal changes. In summer, the asexual individuals were not fully sexualized, though they developed a pair of ovaries. The developing ovaries degenerate if the feeding is stopped. On the contrary, in winter, they developed all the sexual organs. The sexual organs keep developing even if the feeding is stopped after a certain critical point named the point-of-no-return. It was demonstrated that the extreme difference of the sexualization was attributed to the seasonal change of the quality and/or quantity of the sexualizing substance contained in *B. brunnea*, as well as the minor change of the susceptibility to the sexualizing substance in the asexual individuals. On the other hand, the histological research of *B. brunnea* revealed that the degree of the maturation of the sexual organs varied extremely through a year. Taking these results into account, we suggest that the production of the sexualizing substance has no direct relation to any particular mature sexual organs.

Key words: planaria, asexual-sexual switch, sexualization, sexualizing substance, seasonal changes

INTRODUCTION

Asexual reproduction by fission, budding or fragmentation is a widespread phenomenon in most phyla in the animal kingdom including Porifera, Cnidaria, Ctenophora, Platyhelminthes, Rhynchocoela, Endoprocta, Bryozoa, Phoronida, Sipuncula, Annelida, Echinodermata, Hemichordata and Chordata (Bell, 1982). After asexual reproduction, the animals undergo epimorphosis and/or morphallaxis to regenerate into a whole body. In general, the sexual organs are either lacking or undeveloped in the asexual individuals. They may produce gametes depending upon the environmental conditions and/or the phase of life cycle, and eventually undergo sexual reproduction. In the colonial green flagellate *Volvox*, heat shock elicits production of a sexual inducer, a glycoprotein of 30 kDa, and as a result, the asexual individuals develop gametes (Starr and Jaenicke, 1974; Kirk and Kirk, 1986; Mages *et al*., 1988). In metazoans, however, the mechanism underlying the switch from the asexual to the sexual reproduction is poorly studied and the chemicals responsible for the switch have not been isolated yet. In many freshwater planarians, the asexual individuals do not have sexual organs and undergo transverse fission, while the sexual ones have hermaphroditic sexual organs and copulate and then produce cocoons containing several fertilized eggs. Some populations within the same species develop the sexual organs during the colder months of the year, but, when the breeding season is over, the sexual organs degenerate and they continue to fission during the warmer months (Curtis, 1902; Hyman, 1939). Although one of the factors for this alternation seems to be the change of temperature, it is likely that the realization is difficult in a laboratory. The switch from the asexual to the sexual reproduction, namely sexualization, has been attempted by various means other than temperature change. Kenk (1941) and Okugawa (1957) transplanted a piece of sexual body into the asexual individuals. As a result, the sexual organs developed in a part from the asexual individuals. Then, Grasso

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and Benazzi (1973) found that even the exclusively asexual individuals are sexualized, if they are fed with sexual individuals of the same, as well as different, species. These results suggest that sexual individuals contain a putative sexualizing substance(s) of poor species-specificity, which is the first candidate for the chemical(s) responsible for switching from the asexual to the sexual reproduction in metazoans. Although the existence of the sexualizing substance was confirmed by Grasso and Benazzi in 1973 as described above, almost nothing was known about the sexualizing substance itself. This was ascribed to the insufficiency of a method for analyzing the sexualizing process triggered by the sexualizing substance because of the absence of a reliable and relatively quick assay system. Recently, in order to isolate and identify the sexualizing substance, we established an assay system for the sexualization in the OH strain, an exclusively fissiparous strain, of *Dugesia ryukyuensis* by feeding them with fresh worms of *Bdellocephala brunnea*, an exclusively oviparous species (Kobayashi *et al.,* 1999). We divided the process of the sexualization into five distinct stages by histological changes (Kobayashi and Hoshi, 2002). The time required for the sexualization in the assay system was much shorter than ever reported. However, our preliminary experiment throughout a year showed that in only the colder months, especially from December to April, all the worms in the OH strain were fully sexualized at such a speed. Although this unstable result of the sexualization is a serious problem for the purification of the sexualizing substance, from the point of view of zoology, this is an important phenomenon that is to include new information on the sexualizing substance, as well as the process. In fact, this observation suggests that the sexualization has a seasonal change(s).

Meanwhile, the sexualization has a point-of-no-return between stages 2 and 3 (Kobayashi *et al.,* 1999; Kobayashi and Hoshi, 2002). Before the point-of-no-return, the worms in the OH strain return to being asexual if the feeding on *B. brunnea* is stopped. On the contrary, after the point-of-no-return, they are fully sexualized without feeding on *B. brunnea*. Furthermore, it was suggested that the worms after the point-of-no-return produce enough of an amount of their own sexualizing substance and keep the sexuality depending upon the sexualizing substance (Kobayashi *et al*., 2002). Therefore, the time required to be sexualized until the point-of-no-return would be dependent upon the following possibilities: (1) the quality and/or quantity of the sexualizing substance contained in *B. brunnea*; (2) the susceptibility to the sexualizing substance in the asexual individuals. In the exclusively sexual species like *B. brunnea*, generally, the sexual organs develop after hatching from a cocoon. A pair of ovaries, testes, a copulatory apparatus and yolk glands appear in order gradually like the experimental sexualization (Kobayashi *et al.,* 1999; Kobayashi and Hoshi, 2002). A genital pore is a part of the copulatory apparatus. In the assay system, the worms of *B. brunnea* with a genital pore, namely the adults, were used as the source of the sexualizing substance. The other sexual apparatuses are not externally visible because of their opaque body (Fig. 1). In other words, we cannot externally confirm the degree of the maturation, which appears to vary seasonally since the breeding season is restricted within the colder months (Kawakatsu *et al*., 1967; Sakurai, 1998). This is mostly the case with other sexual species as well as *B. brunnea*. The production of the sexualizing substance may be closely related to the maturation in the sexual planarians, because the asexual individuals are not sexualized when they are fed with infants of *B. brunnea* and other asexual individuals (Sakurai, 1981; Kobayashi *et al.,* 1999). Although the predicted seasonal difference of the maturation implies the seasonal change of the quality and/or quantity of the sexualizing substance in the sexual species, we cannot deny the change of the susceptibility to the sexualizing substance as an explanation for the unstable result of the sexualization. Indeed, in the polychaete, *Arenicola marina*, the seasonal reproductive behavior (spawning of gametes) triggered by pheromonal and hormonal signals is controlled by not only zeitgeber but also endogenous rhythm. Even though they are maintained in a constant laboratory condition, the annual reproductive behavior strictly sympathizes with that in natural habitats (Howie, 1963; Hardege *et al*., 1997). In the present study, we clearly demonstrated that the experimental sexualization has seasonal changes that are mostly triggered by the seasonal change of the quality and/or quantity of the sexualizing substance contained in *B. brunnea*. Thus, the histological research in the adults of *B. brunnea* allowed us to discuss the relationship between the sexual organs and the sexualizing substance.

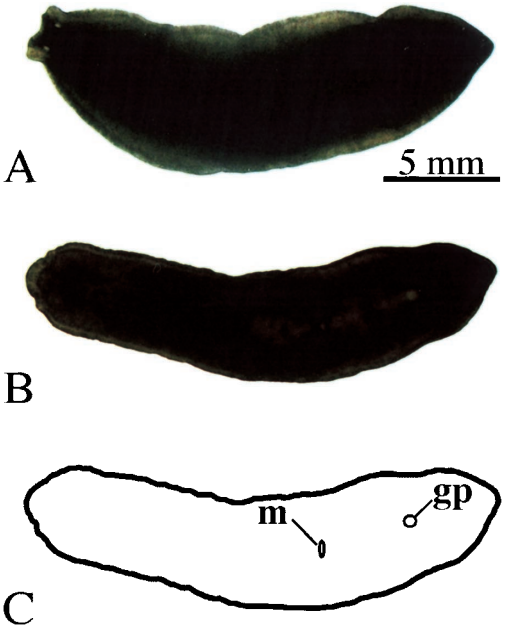


Fig. 1. External views of the adult of *Bdellocephala brunnea* used as the source of the sexualizing substance. The figures are arranged to the anterior on the left. **A**: dorsal view, **B**: ventral view, **C**: schematic drawing of the ventral view. “m” and “gp” represent a mouth and a genital pore, respectively.

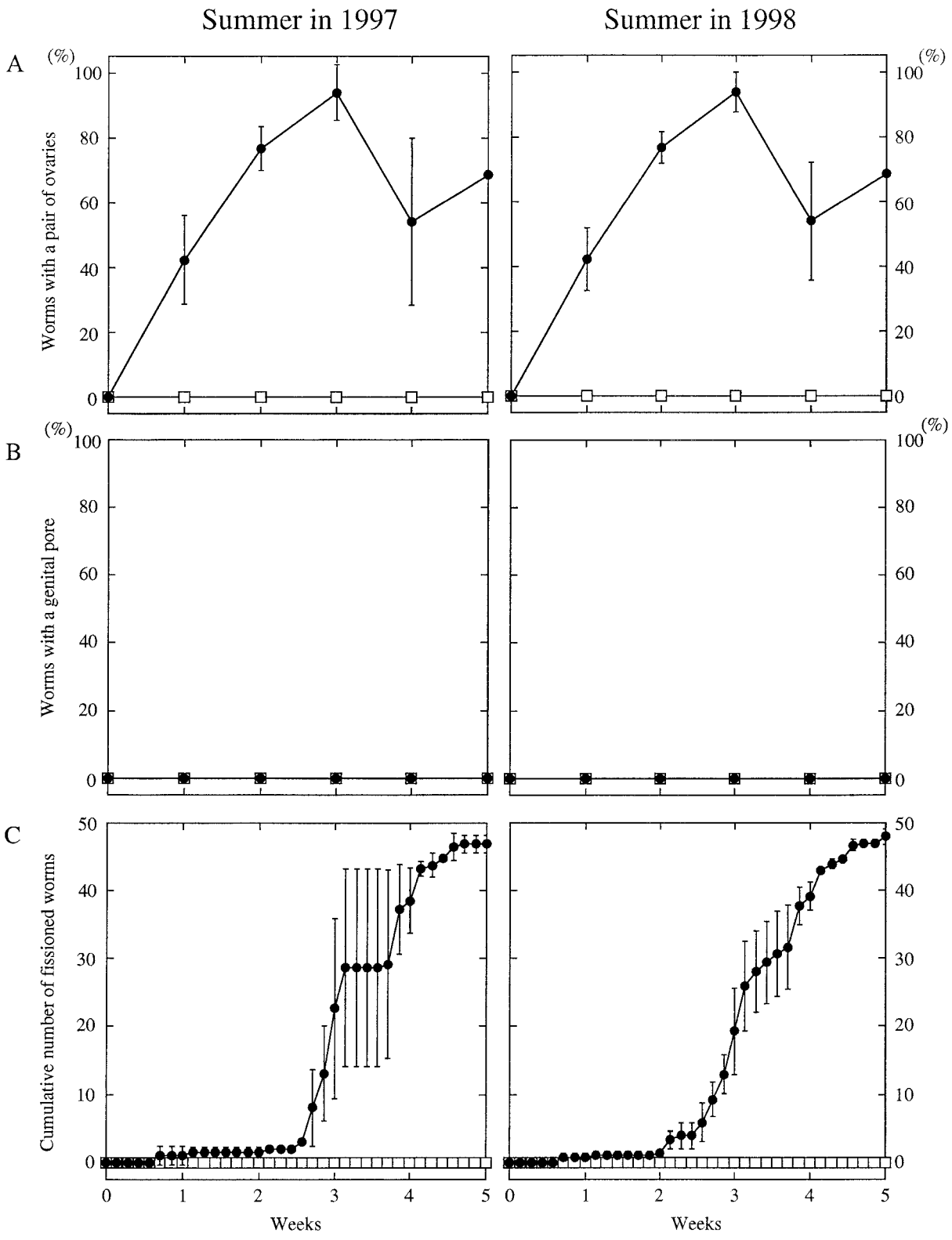


Fig. 2. The sexualization by being fed with fresh worms of *B. brunnea* collected in summer. The number of worms with a pair of ovaries **(A)**, the number of worms with a genital pore **(B)** and the number of fissioned worms **(C)** along with the sexualization. ●, the recipients fed with *B. brunnea*; □, those fed with chicken liver as a control. Two groups of fifty worms of the OH strain were fed with the food for five weeks. During the feeding experiment, second fission in the fissioned worms was not observed. Thus, the total number of fission in the populations is expressed as the cumulative number of the fissioned worms. Means and standard errors (vertical bar) of the two groups are shown.

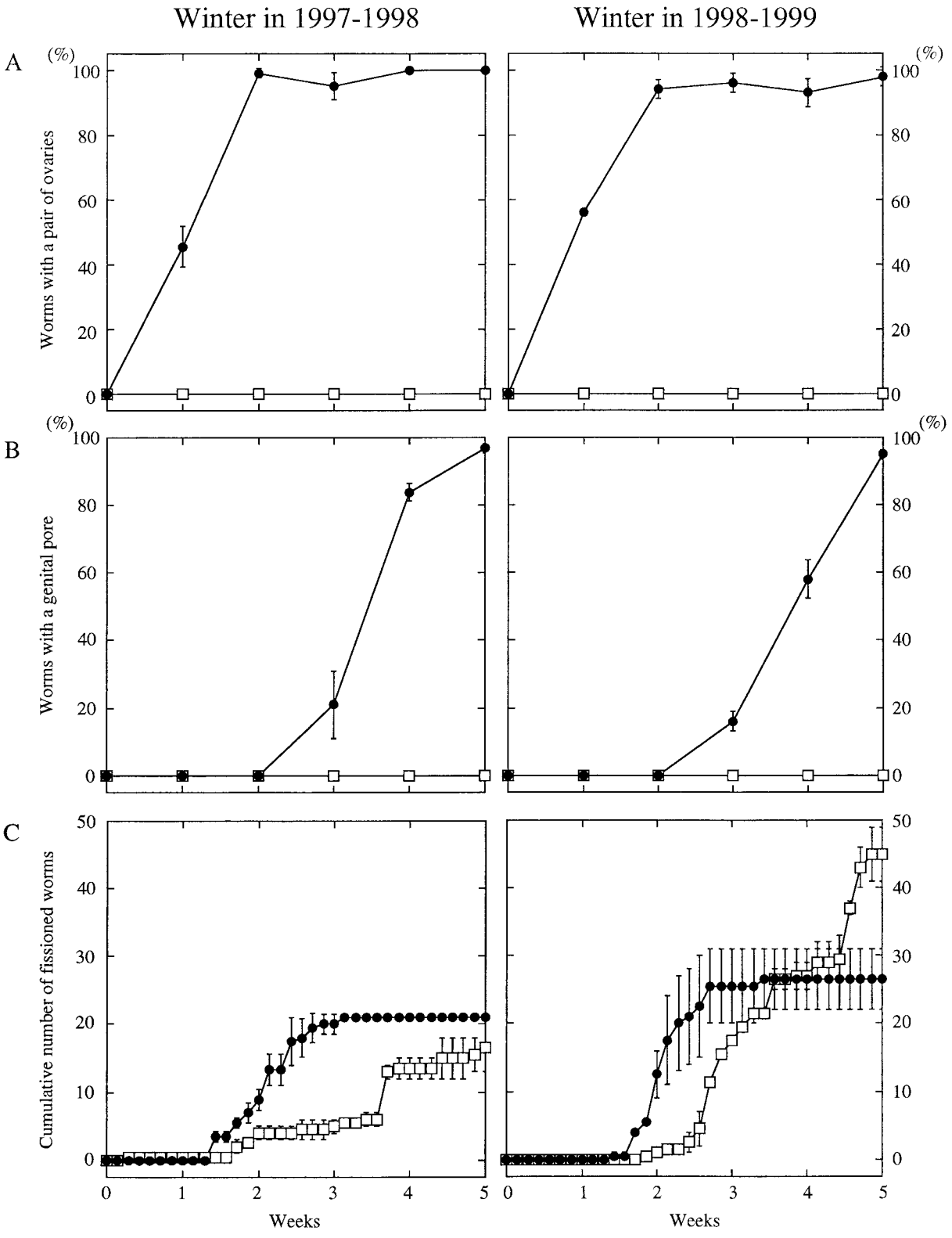


Fig. 3. The sexualization by being fed with fresh worms of *B. brunnea* collected in winter. The number of worms with a pair of ovaries **(A)**, the number of worms with a genital pore **(B)** and the number of fissioned worms **(C)** along with the sexualization. For explanation of symbols, see Fig. 2. Two groups of fifty worms of the OH strain were fed with the food for five weeks. Means and standard errors (vertical bar) of the two groups are shown.

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. After starvation for two to three weeks, the worms were used as asexual recipients for a feeding experiment. Wild populations of *Bdellocephala brunnea*, an exclusively oviparous planaria, were collected in the vicinities of Yamagata City, Japan. The worms with more than 20 mm in body length, as well as with a genital pore, were used as the food to sexualize the worms of *D. ryukyuensis* (Fig. 1).

Feeding experiment

The feeding experiment was performed as previously described (Kobayashi *et al.*, 1999). Two groups of fifty worms of the OH strain were fed with minced fresh worms of *B. brunnea* everyday for five weeks. The experiments over two consecutive years were carried out from 2 June to 7 July 1997 and from 1 June to 6 July 1998 as the feeding in summer, and from 4 December 1997 to 8 January 1998 and from 1 December 1998 to 5 January 1999 as the feeding in winter. According to the condition of temperature in the natural habitat, the worms of *B. brunnea* collected just prior to the experiment were maintained at 25°C in summer and 4°C in winter during the experiment. Furthermore, fifty worms of the OH strain were fed with frozen worms of *B. brunnea* for five weeks. The worms of *B. brunnea* were collected on 4 March and 9 August 1999 as the food in winter and in summer, respectively, and then frozen by liquid nitrogen and stocked at –80°C. This experiment was carried out from 7 July to 14 August 2000 and from 9 January to 13 February 2001. External observation was carried out under a binocular microscope every week to examine whether or not the recipients developed sexual organs (a pair of ovaries and a genital pore). Fission in the population was examined everyday. In case of fission in the recipients, the tail fragments were removed to keep the population density (five worms in about 30 ml of dechlorinated tap water), because the population density effects the process of sexualization (Kobayashi and Hoshi, unpublished data).

Histology

Planarians were fixed with 10 % formalin in 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 fixed specimens were dehydrated through ethanol series, cleared in xylene and embedded in Paraplast Plus™ (Sherwood Medical, St. Louis, MO). The embedded specimens were cut into sections at 4 μm thickness and stained with hematoxylin and eosin.

RESULTS

Sexualization by feeding with fresh worms of *B. brunnea*

In order to demonstrate whether or not there is a seasonal change(s) in sexualization, we need to carry out the examination over, at least, two consecutive years. Accordingly, we carried out the feeding experiment in summer and winter in accordance with the result of the preliminary experiment.

In the case of the feeding in summer, no sexual organs and fission were recognized in the worms of the OH strain, when they were fed on chicken liver which is the daily food to maintain planarians (Figs. 2A–2C, □ and 4G). On the contrary, almost all the worms of the OH strain developed a pair of ovaries by Week 3, when fed with *B. brunnea* (Figs. 2A, ● and 4A). They started to undergo fission actively from the second week of the treatment in spite of the sexualization, and almost all of them experienced fission at the end of the experiment (Fig. 2C, ●). The worms with a pair of developing ovaries underwent fission at the pre- or postpharyngeal level. In other words, they formed head regenerants without or with the pharynx respectively. The regenerants without pharynx degenerated the ovaries until they regenerated a functional pharynx to feed on *B. brunnea*, whereas the regenerants with the pharynx kept their ovaries. As a result, the number of worms with ovaries transiently decreased at Week 4. Although a pair of ovaries were recognized, a genital pore never developed (Figs. 2B, ● and 4A–4C). Histological research of the worms at Week

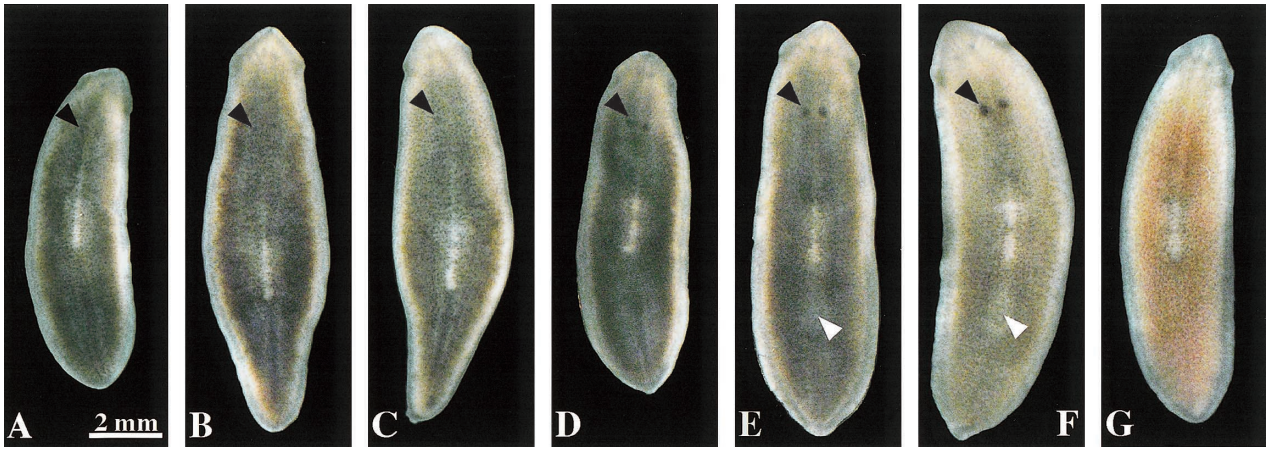


Fig. 4. Anatomical differences in the experimental sexualization between summer and winter. The recipients fed with the fresh worms of *B. brunnea* collected in summer **(A–C)**. **A**, at 3 weeks of the treatment; **B**, at Week 4; **C**, at Week 5. The recipients fed with the fresh worms of *B. brunnea* collected in winter **(D–F)**. **D**, at 3 weeks of the treatment; **E**, at Week 4; **F**, at Week 5. A pair of ovaries and a genital pore are indicated by black and white arrowheads, respectively. The recipient fed with chicken liver as a control for five weeks **(G)**. No sexual organs were externally recognized in the recipients of the control both in summer and in winter. All images are ventral view, the same magnification and arranged to the anterior on the top.

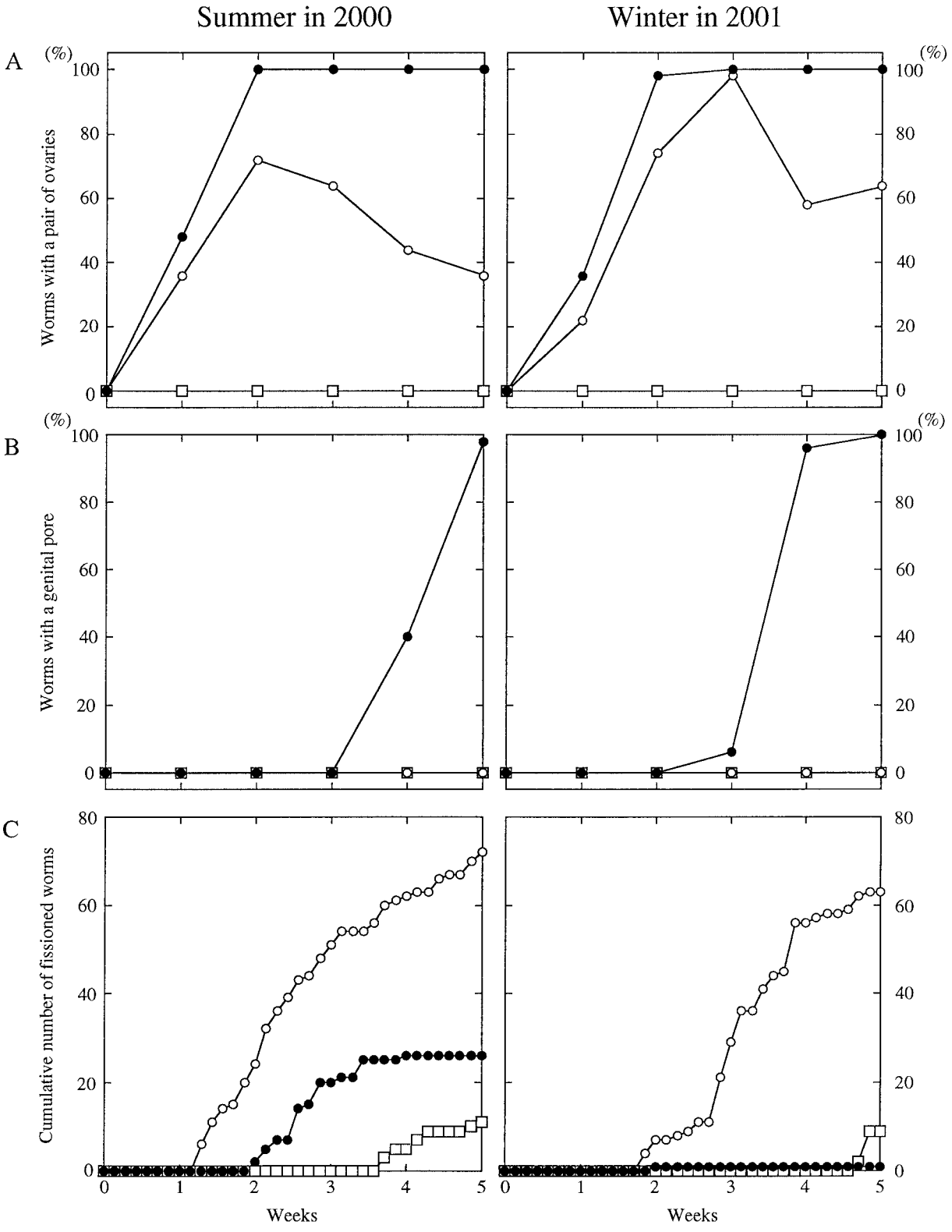


Fig. 5. The sexualization by being fed with frozen worms of *B. brunnea*. The number of worms with a pair of ovaries **(A)**, the number of worms with a genital pore **(B)** and the number of fissioned worms **(C)** along with the sexualization. ○, the recipients fed with the frozen worms of *B. brunnea* collected in summer; ●, those fed with the frozen worms of *B. brunnea* collected in winter; □, those fed with chicken liver as a control. Fifty worms of the OH strain were fed with the food for five weeks. During the feeding experiment, second fission in the fissioned worms was observed. Thus, the cumulative number of the fissioned worms exceeded the total number of the recipients.

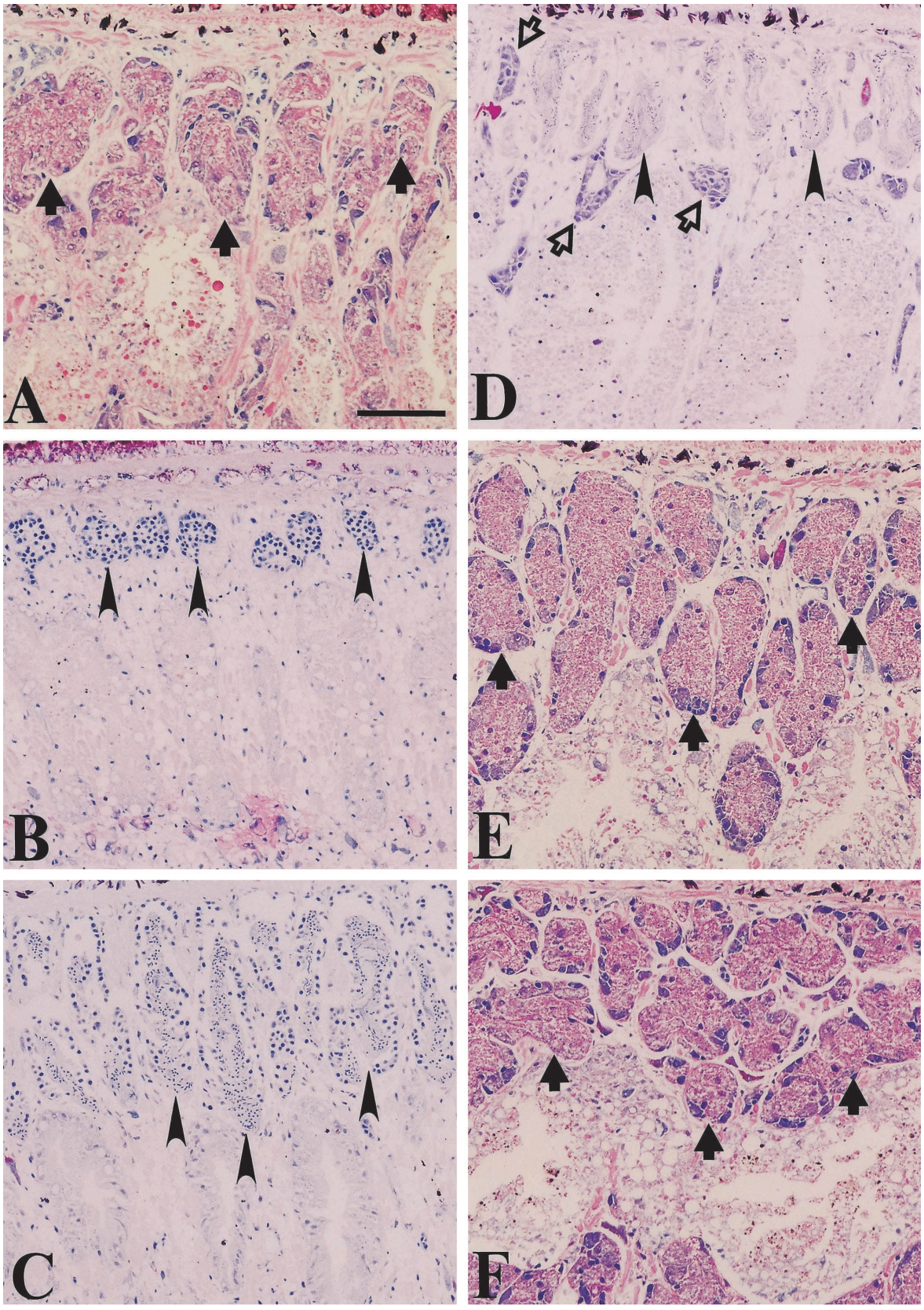


Fig. 6. Morphological change of the testes and the yolk glands in the adults of *B. brunnea* throughout a year. The sections at the same dorsal position in the worms fixed every two months are shown as follows: **(A)** in June, **(B)** in August, **(C)** in October, **(D)** in December, **(E)** in February and **(F)** in April. The closed and open arrows represent a group of mature yolk glands and that of primordial ones, respectively. An arrowhead represents testis. All images are the same magnification and arranged to the dorsal side on the top. Scale bar represents 100 μm.

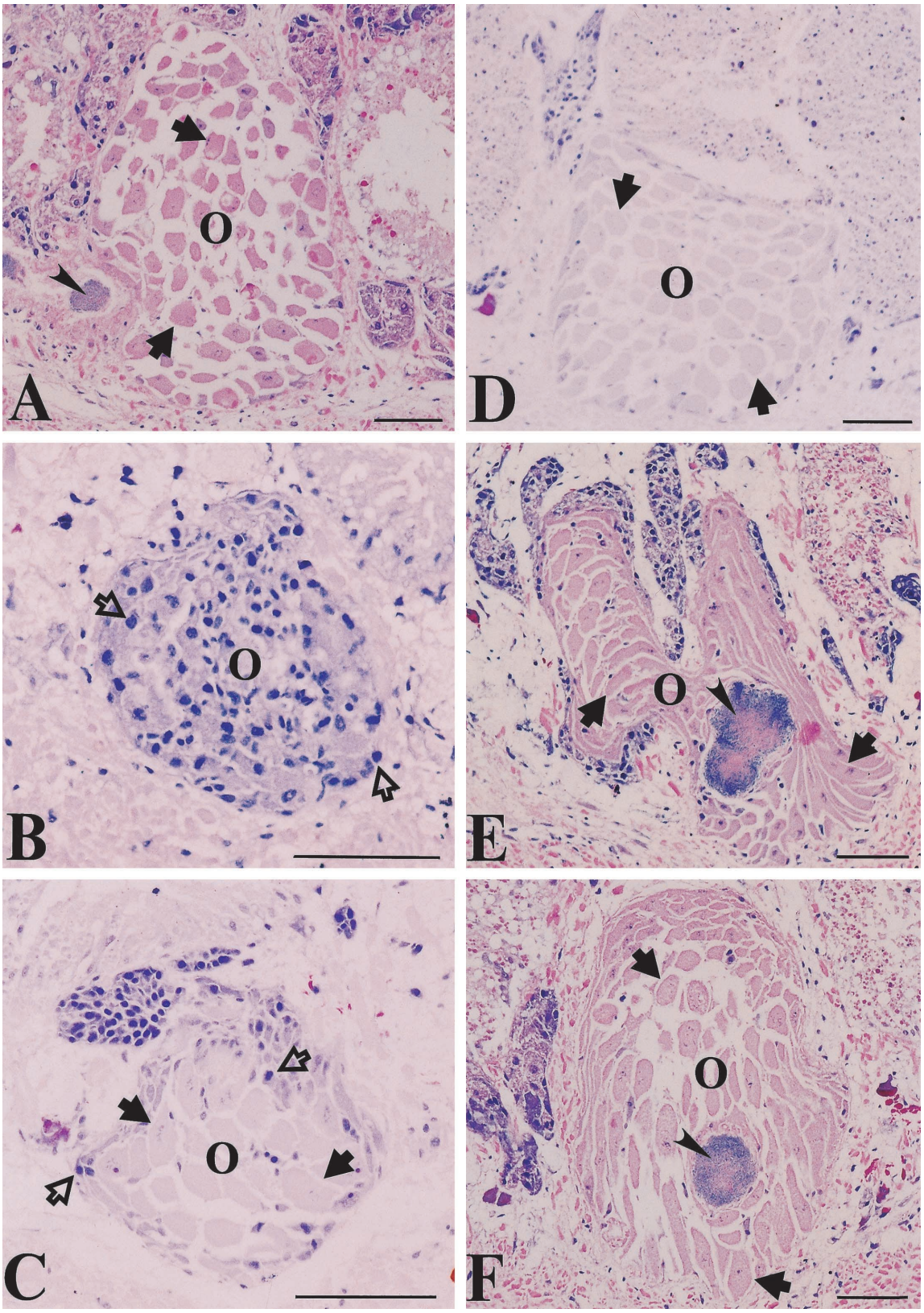
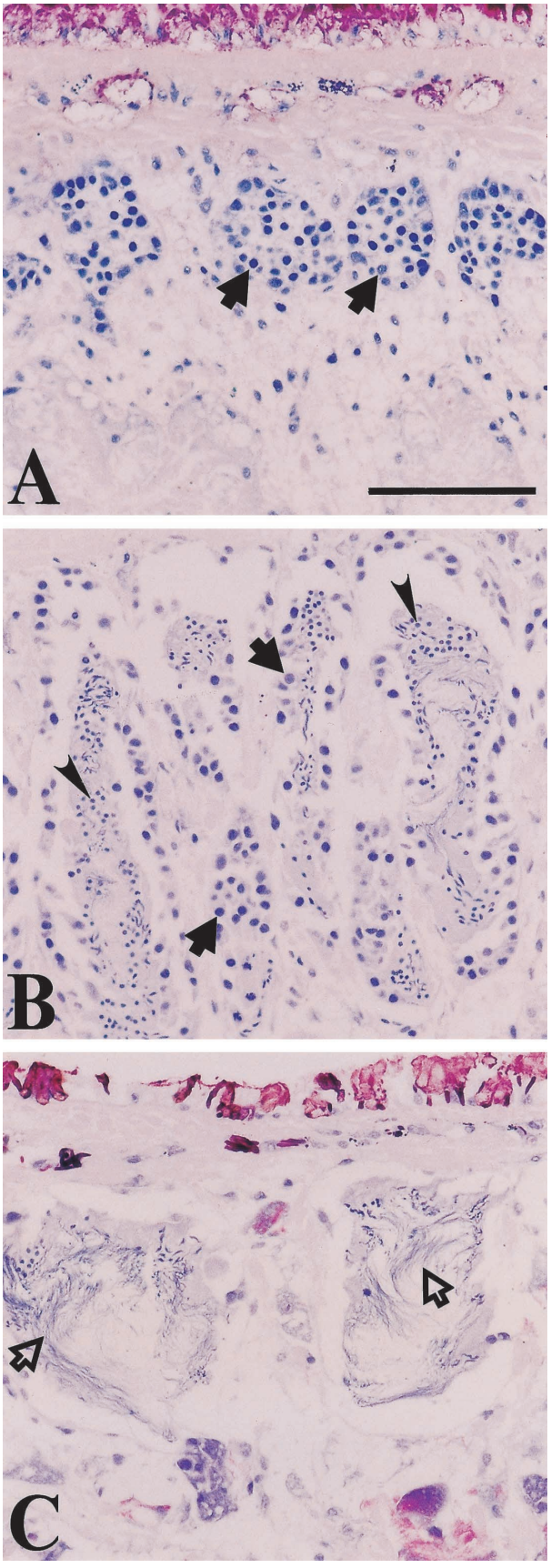


Fig. 7. Morphological change of the ovaries in the adults of *B. brunnea* throughout a year. The largest sections of the ovaries in the worms fixed every two months are shown as follows: **(A)** in June, **(B)** in August, **(C)** in October, **(D)** in December, **(E)** in February and **(F)** in April. Each ovary is indicated as "O". The closed and open arrows represent an oocyte and an oogonium, respectively. An arrowhead represents a group of sperm derived from the other individuals within an oviduct. All images are arranged to the dorsal side on the top. Scale bars represent 100 µm.



5 showed that oocytes appeared in the ovaries, yet other sexual organs were not detectable.

In the case of the feeding in winter, the worms of the OH strain developed not only a pair of ovaries but also a genital pore and were fully sexualized by Week 5, when fed with *B. brunnea* (Figs. 3A, 3B, ● and 4D–4F). At two weeks of the treatment, almost all of them developed a pair of ovaries, and then kept developing them in contrast with the undeveloped ovaries in summer (Fig. 4A–4F). Though they also started to undergo fission actively from the second week of the treatment, they mostly ceased undergoing the fission at Week 3 (Fig. 3C, ●). In the case of the fission, most of them underwent fission at the post-pharyngeal level. Even though a few of them underwent fission at the pre-pharyngeal level, the regenerants without pharynx did not degenerate a pair of developing ovaries. Histological research of the worms at Week 5 showed that all the sexual organs were mature. On the contrary, no sexual organs were recognized in the worms of the OH strain, when they were fed on chicken liver in winter as well as in summer (Figs. 3A, 3B, □ and 4G). The control worms in winter underwent fission from about two weeks of the treatment (Fig. 3C, □). Although, as described above, the fission in worms fed with *B. brunnea* kept stopping after Week 3, it was notable that the fission started faster than that in the control worms (Fig. 3C). As a result, the progression of the sexualization in the feeding treatment between summer and winter was quite different. There was no significant difference in duplicated experiments over two years.

Sexualization by feeding with frozen worms of *B. brunnea*

We also carried out the experimental sexualization by feeding worms in the OH strain with frozen worms of *B. brunnea*. In the case of the feeding with *B. brunnea* frozen in summer, worms of the OH strain developed a pair of ovaries but not a genital pore both in summer and in winter like the case of feeding with the fresh worms in summer (Fig. 5A, 5B, ○). They also continued to undergo fission actively (Fig. 5C, ○). However, between summer and winter, there were several slight differences with respect to the number of the worms with a pair of ovaries. As a whole, the number of the worms with a pair of ovaries in summer was fewer than that in winter (Fig. 5A, ○). The number of fissioned worms at the pre-pharyngeal level was mostly similar in summer (58.3%) and winter (71.4%). In the case of the feeding with *B. brunnea* frozen in winter, worms of the OH strain devel-

Fig. 8. Morphological change of the testes at high magnification. **(A)** in August, **(B)** in October and **(D)** in December. A closed arrow represents spermatogonium or a spermatocyte. In general, it is believed that the cells with a condensed nucleus in the testes are spermatocytes. The arrowhead and open arrow represent a spermatid and a group of sperm, respectively. All images are the same magnification and arranged to the dorsal side on the top. Scale bar represents 100 μm.

oped not only a pair of ovaries but also a genital pore both in summer and in winter like the case of feeding with the fresh worms in winter (Fig. 5A, 5B, ●). About one third of them underwent fission in summer, whereas almost all of them did not in winter (Fig. 5C, ●). The fission in summer mostly kept stopping after Week 3 like the case of feeding with the fresh worms in winter. In the case of the feeding with chicken liver, no sexual organs were recognized in the worms of the OH strain both in summer and in winter (Fig. 5A, 5B, □). The worms fed with chicken liver hardly underwent fission (Fig. 5C, □). As described in the previous section, the control worms in winter underwent fission actively, whereas those in summer did not (Figs. 2C,□ and 3C, □). In this experiment, the cycle of fission in the control worms was apparently different from that in the worms of previous control group.

Histological research in adults of *B. brunnea*

We carried out the histological research in the worms of *B. brunnea* as described in MATERIALS AND METHODS, which were collected every two months from August 2000 to June 2001. In February, April and June, yolk glands were mature and located throughout the whole body other than the head (Fig. 6A, 6E, 6F), whereas testes were entirely lacking. Although a pair of ovaries were mature with a number of oocytes from February to June, the oocytes were loosely distributed, gradually (Fig. 7A, 7E, 7F). Numerous sperm were observed in a seminal receptacle and a pair of seminal vesicles in spite of the defect of the testes, and they also decreased gradually. In August, all the sexual organs were immature. Neither oocytes nor sperm were detectable in a pair of ovaries and testes respectively (Figs. 7B and 8A). Yolk glands were entirely lacking (Fig. 6B). In October, oocytes and spermatids became detectable in a pair of ovaries and testes respectively (Figs. 7C and 8B), but yolk glands were not apparent yet (Fig. 6C). In December, a pair of ovaries were mature and primordial yolk glands appeared (Figs. 6D and 7D). Sperm mostly occupied the testes (Fig. 8C).

DISCUSSION

The feeding experiment by being fed with fresh worms of *B. brunnea* over two consecutive years, demonstrated that there are seasonal changes in the experimental sexualization (Figs. 2 and 3). In summer, though the worms in the OH strain developed a pair of ovaries, they underwent fission actively (Fig. 2C, ●). The decrease in the worms with the ovaries was attributed to the regenerants without pharynx. They transiently degenerated a pair of ovaries, because they could not take in the sexualizing substance contained in *B. brunnea* until they regenerated a functional pharynx (Fig. 2A, ●). This means that the head fragments immediately after fission had not acquired the sexuality yet; that is to say, they did not overcome the point-of-no-return. Although the tail fragments after fission were removed to keep the population density, it appeared that they also did not overcome the point-of-no-return since they regenerated to become asexual (data not shown). On the other hand, in the intact worms and the regenerants with pharynx, a pair of ovaries were always observed after Week 3 but the size did not externally grow (Fig. 4A–4C). Furthermore, the worms in the OH strain never developed a genital pore (Fig. 2B, ●), which is one of the morphological features after the point-of-no-return. In addition to the external morphology, the histological research of the worms at Week 5 showed that they were sexualized by stage 2. These results indicate that the worms in the OH strain cannot overcome the point-of-no-return by the feeding with fresh worms of *B. brunnea* in summer, though they developed a pair of ovaries with several oocytes. Such results were obtained from early June to late September (data not shown). On the contrary, in winter, the worms in the OH strain kept developing a pair of ovaries externally (Fig. 4D–4F). Although the fission was observed from two to three weeks of the treatment, it kept stopping after Week 3 (Fig. 3C, ●). At 5 weeks of the treatment, most of them opened a genital pore (Figs. 3B, ● and 4F). The histological research of the worms at Week 5 showed that they were sexualized by stage 5. These results indicate that the worms in the OH strain can overcome the point-of-no-return by the feeding with fresh worms of *B. brunnea* in winter, and agree well with our previous studies (Kobayashi *et al.,* 1999; Kobayashi and Hoshi 2002). Such results were obtained from early December to late March (data not shown). After the fission, the regenerants without pharynx did not degenerate a pair of ovaries (Fig. 3A, ●). This suggests that the worms in the OH strain had already overcome the point-of-no-return from two to three weeks of the treatment.

What caused the extreme difference in the time required to be sexualized until the point-of-no-return? As described in INTRODUCTION, the time required to be sexualized until the point-of-no-return would be dependent upon the quality and/or quantity of the sexualizing substance contained in *B. brunnea*, and/or the susceptibility to the sexualizing substance in asexual worms in the OH strain. In order to examine this problem, we fed the worms in the OH strain with the frozen worms of *B. brunnea*. Indeed, we had to pool the sexual planarians to purify the sexualizing substance, because we cannot expect that they contained the sexualizing substance in a large quantity. Fortunately, the preliminary experiment revealed that the frozen worms were effective as well as live ones. We clearly showed that worms in the OH strain could not overcome the point-of-no-return whenever fed with *B. brunnea* frozen in summer, while they were fully sexualized whenever fed with *B. brunnea* frozen in winter. Histological research revealed that the worms fed with *B. brunnea* frozen in summer and winter were sexualized by stages 2 and 5, respectively. Therefore, we conclude that the seasonal changes are mostly triggered by the seasonal change of the quality and/or quantity of the sexualizing substance contained in *B. brunnea*. However, there

were several slight differences between summer and winter as follows. In the case of the feeding with *B. brunnea* frozen in summer, the number of the worms with a pair of ovaries in summer was fewer than that in winter, especially at Week 3 (Fig. 5A, ○). Since the number of fissioned worms at the pre-pharyngeal level was mostly similar in summer and winter, the decrease in the worms with a pair of ovaries in summer is not attributed to the increase in the regenerants without pharynx, which transiently degenerated the ovaries. Thus, this suggests in the OH strain that the susceptibility to the sexualizing substance contained in *B. brunnea* frozen in summer differs between summer and winter. On the other hand, in the case of the feeding with *B. brunnea* frozen in winter, about half of the worms in the OH strain underwent fission in summer, whereas almost all of them did not in winter (Fig. 5C, ●). As a result of the fission, the appearance of a genital pore in summer was delayed in comparison with that in winter, because the fissioned worms transiently lost the region where the genital pore should open (Fig. 5B, ●). Since the cessation of fission occurs immediately after the point-of-no-return (Kobayashi and Hoshi, 2002), the difference in the occurrence of the fission means the difference of the time required to be sexualized until the point-of-no-return. Therefore, this suggests in the OH strain that the susceptibility to the sexualizing substance contained in *B. brunnea* frozen in winter also differs between summer and winter. Accordingly, we presume that the seasonal changes of the sexualization are also affected by the change of the susceptibility to the sexualizing substance in the asexual individuals. The difference of the cycle of fission observed in the control worms (Figs. 2C, □, 3C, □ and 5C, □) implies that fission is controlled by a rhythm other than the seasonal changes of sexualization.

The seasonal change of the quality and/or quantity of the sexualizing substance caused the extreme difference in whether or not worms in the OH strain overcame the point-of-no-return. If the seasonal change of the quality of the sexualizing substance occurred, the worms of *B. brunnea* would contain an "ovarian developing substance" besides a "true" sexualizing substance responsible for the point-of-no-return, since the feeding with *B. brunnea* frozen in summer induced merely a pair of ovaries with several oocytes in spite of the long-term feeding. To examine this possibility further, studies should be undertaken on the purification of the sexualizing substance. If so or not, the extreme histological changes in *B. brunnea* (Figs. 6–8) allowed us to discuss the relationship between the sexual organs and the production of the sexualizing substance. We suggested the important points described as follows. At least, a pair of ovaries with several oocytes appear to be induced by being fed on *B. brunnea* without the mature sexual organs as observed in August (Figs. 6B, 7B and 8A), suggesting that the production of the "ovarian developing substance" (or rather, the "true" sexualizing substance) has no relation to any the particular mature sexual organs. We showed, for the first time to the best of our knowledge, an interesting fact that the mature testes do not coexist with the mature yolk glands at the same time in *B. brunnea* (Fig. 6). It was, generally, believed that the sexually mature planarians equip all the mature sexual organs simultaneously. From October to December, when the time required to be sexualized until the point-of-no-return rapidly shortened, the testes and the ovaries became mature gradually in *B. brunnea* (Figs. 7C, 7D and 8B, 8C). Then, from December to April, when all the worms in the OH strain were fully sexualized with almost the same speed, the testes disappeared and by contrast the yolk glands became mature in *B. brunnea* (Fig. 6D–6F). Thus, the production of the "true" sexualizing substance would be independent of both the mature testes and the mature yolk glands. Sakurai (1981) found in *B. brunnea* that even the feeding of pieces without the ovaries was effective for the complete sexualization. A pair of mature ovaries seems to be excluded as the origin of the "true" sexualizing substance. From April to June, although the time required to be sexualized until the point-of-no-return dramatically extended, there was no significant histological changes in *B. brunnea* (Figs. 6A, 6F and 7A, 7F). It is notable that in June, the complete sexualization appears to no longer take place even by the feeding of *B. brunnea* with the mature yolk glands (Fig. 6A). This also suggests that the production of the "true" sexualizing substance is independent of the mature yolk glands. With respect to the production of the sexualizing substance in the sexual individuals, it was, so far, reported as follows. Grasso *et al.* (1975) found a large number of nerve endings containing neurosecretory granules in the two effective fractions obtained from the homogenate of sexual individuals, suggesting that the sexualizing substance is a neurosecretory substance(s). We, at least, presumed that the production of the sexualizing substance is independent of the particular mature sexual organs, because the worms immediately after the point-of-no-return, namely the worms at stage 3 seem to produce enough of an amount of their own sexualizing substance in spite of the absence of the particular mature sexual organs (Kobayashi *et al.*, 2002). The result in the present study is compatible with these possibilities.

Finally, we have to mention that fission of worms in the OH strain was apparently facilitated by being fed with *B. brunnea* (Figs. 2C, 3C and 5C). Although we have already found that *B. brunnea* contains the "fission-inducing substance" besides the sexualizing substance (data not shown), we do not describe this finding in this paper to avoid confusion for the reader.

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