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Gonadal State of Wild Amphioxus Populations and Spawning Success in Captive Conditions during the Breeding Period in Japan

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ABSTRACT—Adult individuals of amphioxus (*Branchiostoma belcheri*) were collected by dredging from a research vessel at selected stations in two areas off the coast of Japan in July 2000: Deyama and Takamatsu so named by local fishermen in the Enshu Nada Sea. The number of males collected exceeded that of females at all the stations in Takamatsu and at four of five stations in Deyama. The over all sex ratio (males : females) of the collected animals was 1.2 : 1. The animals showed various maturational stages of the gonad, and approximately 70% had mature gonads. However, post-spawning animals were identified only at two stations in Takamatsu. Mature animals were placed in laboratory tanks. These animals remained in good conditions for about two months, and many animals spontaneously spawned in the tanks. This is the first report of spontaneous spawning of *B. belcheri* in Japan.

Key words: amphioxus, lancelet, spawning, reproduction, gonad

INTRODUCTION

Amphioxus, subphylum Cephalochordata, phylum Chordate, has been known as an animal group of considerable phylogenic importance among chordates. For more than a century, this species has been the subject of numerous comparative studies attempting to elucidate the evolutionary origin of vertebrates. In Japan, collection of amphioxus (*Branchiostoma belcheri*) was first reported in 1882 (Nishikawa, 1995a). Since the first collection, a number of surveys conducted in amphioxus have revealed that its habitats occur mainly along the Pacific Ocean coasts of southwestern Japan, including coastal areas around Kyushu, and in the Seto Inland Sea (Nishikawa, 1981). Over time, Japanese populations of amphioxus have gradually disappeared throughout Japan, and amphioxus was recently recognized as a locally endangered species. The population in the Oshima in Mikawa Bay, one of these dwindling populations, was thought to have been extinct for about the past twenty years (Nishikawa, 1995b). Fortunately, a population of amphioxus was found recently in the Irago Channel, which is located at the mouth of Mikawa Bay and Ise Bay, and the west end of Enshu-Nada Sea (Kubokawa *et al*., 1998). This population was found to be sufficiently large to supply adult amphioxus for research.

Chin (1941) extensively studied the habitat environment, local distribution, growth, and reproduction of amphioxus in Xiamen, China. The ecology of Japanese populations of amphioxus has been studied in situ or in captivity (Kikuchi, 1977; Kubokawa *et al*., 1998). However, neither has reported any observations of spawning either in the field or in the laboratory.

The breeding season of amphioxus lasts usually from late June until mid August in both Japan and China (Chin, 1941; Kikuchi, 1977; Fang, 1989; Kubokawa *et al*., 1998). However, no Japanese laboratory has succeeded in inducing captive spawning of amphioxus. Japanese biologists wishing to obtain amphioxus embryos had to rely on a supply of fertilized eggs from Florida, US (*Branchiostoma floridae*) and Quingdao, China (*Branchiostoma belcheri*), where fertilized eggs are obtained by electrical stimulation of the animals (Stokes and Holland, 1996) and after spontaneous spawning in a tank after collection (Wu *et al*., 1994),

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respectively.

To clarify the reproductive state of *B. belcheri* from the Enshu Nada Sea, we collected a large number of animals of this species at specific locations in the Enshu Nada Sea areas in July 2000, and investigated the gonad maturational stage of sampled animals. In addition, we reared adult amphioxus in laboratory tanks in July and August 2000, and succeeded in observing spontaneous spawning.

MATERIALS AND METHODS

Sampling of amphioxus

Adult amphioxus (*Branchiostoma belcheri*) were collected in two areas, one in the Irago Channel (Kubokawa *et al.*, 1998) and the other in the open sea off the Atsumi Peninsula, Aichi Prefecture. These two areas are referred to as "Deyama" and "Takamatsu", respectively, by local fishermen (Fig. 1). The approximate latitude and longitude of Deyama and Takamatsu are 34°29'N, 137°04'E and 34°35'N, 137°16'E, respectively. The research vessel Tansei-Maru (610 tons), of the Ocean Research Institute, University of Tokyo, was used for the ecological study of the amphioxus populations on July 1 and 2, 2000. Animals were collected with a cylindrical stainless steel dredge (60 cm in diameter and 90 cm in depth).

Animals collected in July 2000 at five stations in Deyama and four stations in Takamatsu (Fig. 2) were visually examined and classified into four gonadal developmental stage groups: undeveloped, immature, mature, and post-spawning according to Fang and Qi (1990) with some modification. Animals at the undeveloped stage had no visible gonads. Animals at the mature and immature stages had a pair of visible gonads. The gonads of the immature stage animals were small (less than approximately 1 mm in length). In mature animals, males and females were visually distinguishable; the color of the ovary was yellowish white and yellow, whereas the color of the testis was plain white. Animals at the post-spawning stage had small atretic gonads. The sex was visually indistinguishable in the undeveloped, immature, and post-spawning stage animals.

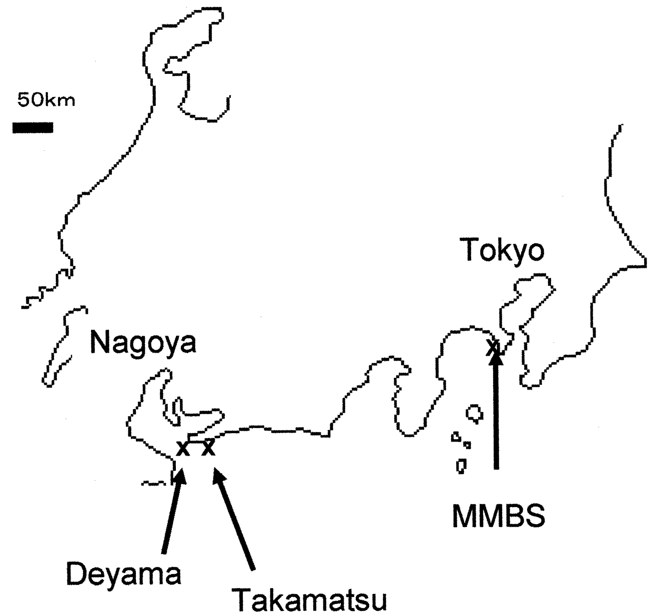

Fig. 1. Location of the two sampling areas, Deyama and Takamatsu, in the Enshu Nada Sea. Collected amphioxus was transported to the Misaki Marine Biological Station (MMBS).

Maintenance of captive amphioxus

Animals collected on July 1 and 2, 2000, were transported to the Misaki Marine Biological Station (MMBS), University of Tokyo, and animals among them that were at the mature gonad stage were kept in four tanks in a room that was isolated from other laboratory rooms. The room has a floor area of approximately 30 square

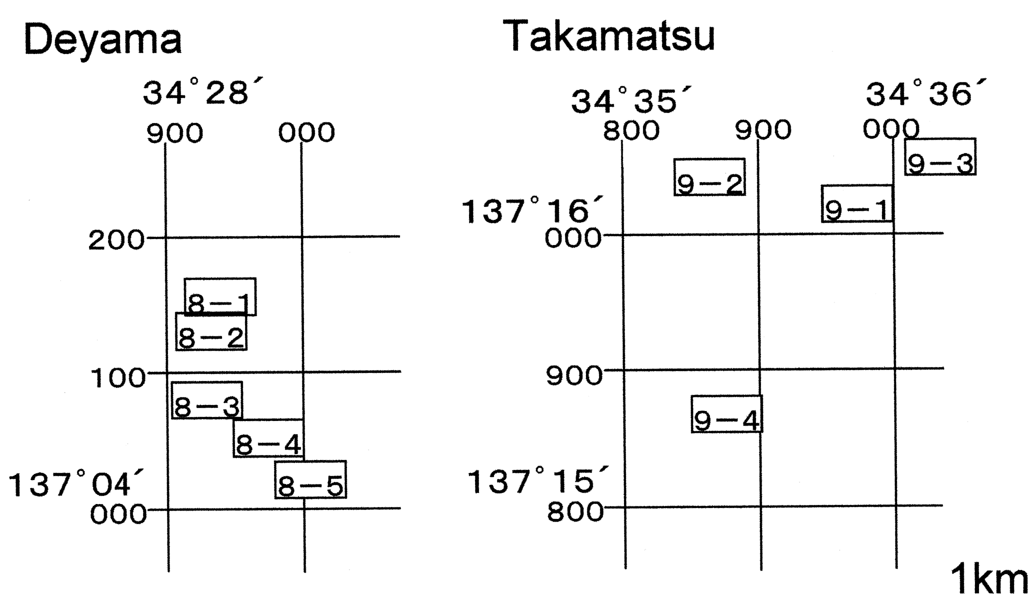

Fig. 2. Location of repeated dredging at narrow areas in Deyama and Takamatsu. The latitude and longitude of the various stations in Deyama (8-1 to 8-5) and Takamatsu (9-1 to 9-4) were as follows: 8-1 (34°28'926 N, 137°04'158 E), 8-2 (34°28'915 N, 137°04'133 E), 8-3 (34°28'907 N, 137°04'093 E), 8-4 (34°28'974 N, 137°04'069 E), 8-5 (34°28'980 N, 137°04'024 E), 9-1 (34°35'957 N, 137°16'030 E), 9-2 (34°35'342 N, 137°16'045 E), 9-3 (34°36'057 N, 137°16'066 E), 9-4 (34°35'887 N, 137°15'871 E). Stations in which no animals were collected were omitted and the numbers of stations were renumbered.

meters and two windows on the east wall and a frosted glass door to the outside on the west. Two of the four tanks were made of opaque black plastic and cylindrical in shape, measuring 43 cm in diameter and 32 cm in depth (tank A and B). The other two tanks were made of transparent acryl, and one (tank C) was rectangular (60 cm long by 30 cm wide by 36 cm deep), the other (tank D) cylindrical (30 cm in diameter and 1 m in depth). All the tanks contained a sand layer 5-cm thick and were filled with seawater.

We placed approximately 200 fully mature females and the same number of fully mature males into tank A on July 3, 2000. The remaining several hundred animals were put into tank B. Tanks C and D were used for recording swimming behavior with a video camera. We also placed approximately 100 fully mature females and the same number of fully mature males into tank C (on July 3) and tank D (on July 27) respectively. Animals in tank D were mature ones sorted out from animals in the tank A, which contained both mature and post-spawning ones. Most of the animals in tank A were post-spawning ones. Then sides of tanks C and D were covered with a black shading sheet from 9:00 to 17:00. Tops of the four tanks were not covered.

Tank water was changed at 9:00 every morning. The room was air conditioned at about 25°C. No artificial illumination was used. Animals were fed with chlorella (*Nannochloropsis* sp.) every morning after the water was changed. When the tank water was changed, the used water was filtrated through a 100-μm nylon mesh to sift our embryos. The presence of embryos was used as evidence of spawning. Animals with regressed gonads were sorted out from these tanks and placed into a separate tank. Gonads of these sorted animals never developed again during the experimental period.

Recording of spawning behavior

Spawning behavior was recorded in tanks C and D under a visually dark condition with a high sensitivity black-and-white video camera (Sony SSC-M) under infrared lamp illumination from 18:00 or 19:00 to 3:00 or 6:00 of the next morning from July 3 until July 30, 2000 in tank C and from July 28 until July 31, 2000 in tank D. A video camera was put on the side of tank C for recording the whole part of the tank. In tank D, three video cameras were used, one each for recording the lower (from the bottom to 30 cm in height), middle (between 30 and 60 cm in height), and upper (between 60 and 90 cm in height) parts of the tank. Spawning time and type of swimming behavior were observed by playing back the videotapes. Differences could not be distinguished between males and females or between spawned eggs and sperms on the video display.

Data analysis

Sex ratios were statistically tested by the one-sided binominal test under the null hypothesis of the 1 : 1 ratio (Campbell, 1974). The χ^2 test for multiple samples (Campbell, 1974) was applied to compare the distribution of the gonad maturational stages between stations or sea areas.

RESULTS

Sex ratio and gonad maturation stage

Sex ratio and the gonad maturational stage were studied in animals collected in Deyama and Takamatsu (Figs. 1 and 2). With regard to sex ratio, males dominated females at 4 of the 5 stations in Deyama and at all of the 4 stations in Takamatsu (Fig. 3A). In terms of the sum of all the stations in each area, the number of males exceeded the number of females: significantly so in Deyama (P=0.028) and in Takamatsu (P=0.038). The sex ratio for the total of both Deyama and Takamatsu was 1.21 (males/females); this value was very significantly different from 1 (P=0.003).

Animals at the undeveloped, immature, and mature stages were found in all stations in both Deyama and Takamatsu, but animals at the post-spawning stage were found only in two stations in Takamatsu and in none of the stations in Deyama (Fig. 3B). The ratios of mature animals of all animals were 70.2% and 63.4% in Deyama and Takamatsu, respectively. The distribution of animals among these gonad maturational stages differed very significantly among the stations in both Deyama (P<0.001 by the χ^2 test among every station except for 8≠3) and Takamatsu (P<0.001 by the χ^2 test among all stations), and also between Deyama and Takamatsu (P<0.001 by the χ^2 test) (Fig. 3B). Although the presence of sexually undistinguishable animals should influence the sex ratio, such animals were only 1.6 to 2.3%, and therefore the influence can be neglected.

Observation of spontaneous spawning in the tank

Spontaneous spawning occurred in all the tanks (Fig. 4). It started on July 13 and continued with zero to two-day intervals until July 21 in tank A. It started on July 14 with zero to two-day intervals until July 24, with additional spawning on August 5 and 6 after a long interval, in tank B.

Tank C was covered with a black sheet through the daylight hours on July 19. On the following day, animals in this tank started spawning, and daily spawning continued until July 23 with additional spawning on July 31 and August 10. Tank D received adult animals on July 27 and was immediately covered with a black sheet. Animals in this tank spawned on the following two days and on July 31. The cover in tank C and D was used in the day time during the observation. The spawning dates did not correlate with the lunar cycle (Fig. 4).

Swimming behavior of amphioxus in the tank

Daily total numbers of animals that emerged from the bottom and swam in tank C are shown in Fig. 5. The swimming behavior was first observed on July 9 and then, after 7 inactive days, on every day from July 17 until July 26. The spawning was recognized on 4 successive days from July 20 around the peak of swimming activity. No spawning was detected on the other days.

In tank D, the swimming behavior was observed from July 28 until July 31. The spawning was recognized on these three days. The majority of emerged animals swam up to the water surface, whereas the remaining animals ceased swimming upward before reaching the water surface in the tank of 1m deep and returned to the sandy layer at the bottom (Fig. 6). In both tanks, some animals swam along just beneath the water surface for more than several seconds after reaching it (data not shown).

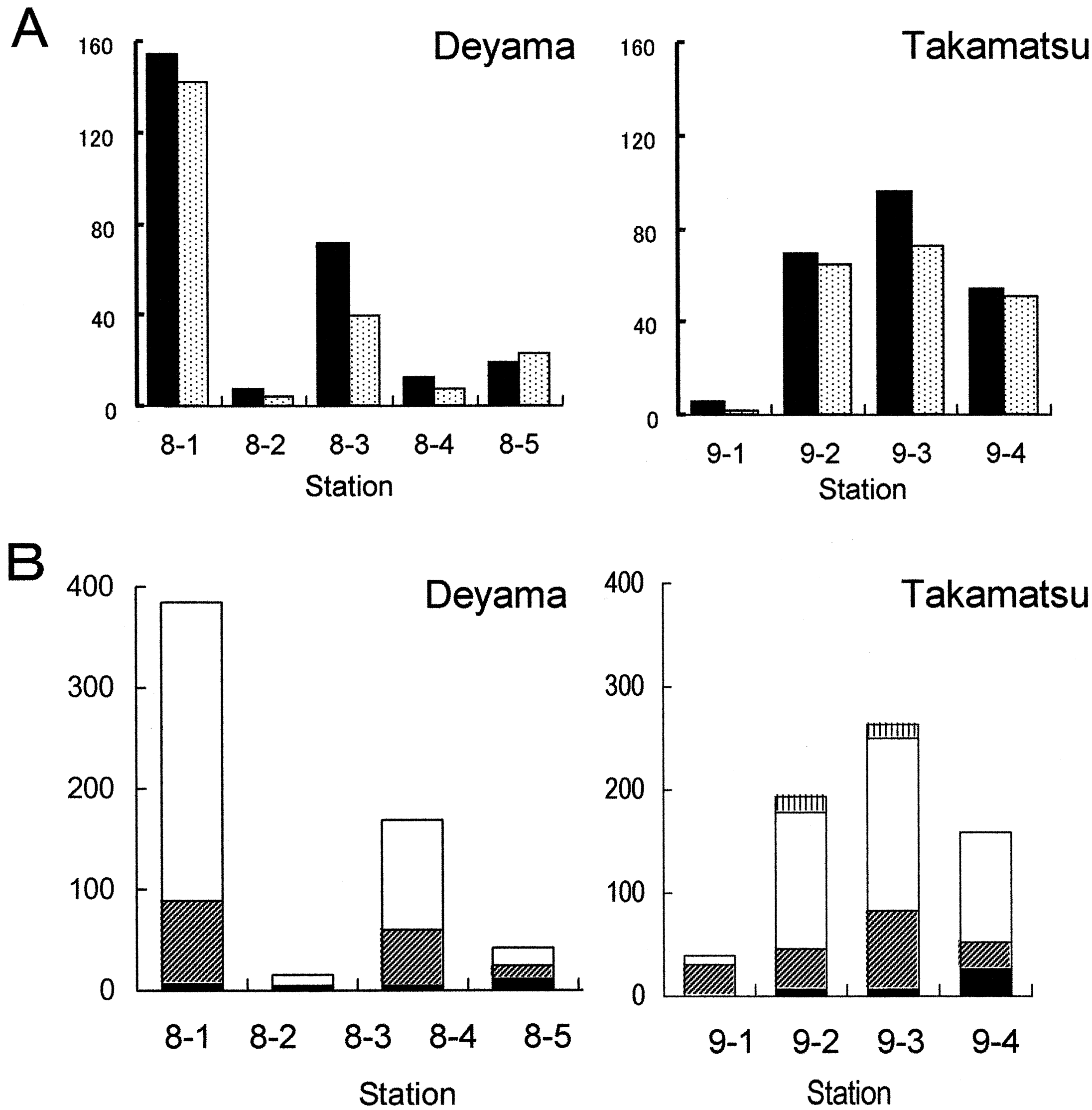

Fig. 3. The sex ratio and differences of reproductive conditions in amphioxus (*Branchiostoma belcheri*) in stations in Deyama and Takamatsu. (A) The number of males (solid column) and females (dotted column) in neighboring patches in Deyama and Takamatsu populations. (B) The number of animals with conditions of post-spawning (striped column), mature (open column), immature (shaded column) and undeveloped (solid column) stages in neighboring stations in Deyama and Takamatsu. In Deyama the number of amphioxus were 385, 16, 169, 43, and 68 in 8-1, 8-2, 8-3, 8-4, and 8-5, respectively. In Takamatsu, the numbers were 39, 194, 262, and 158 in 9-1 9-2, 9-3, and 9-4.

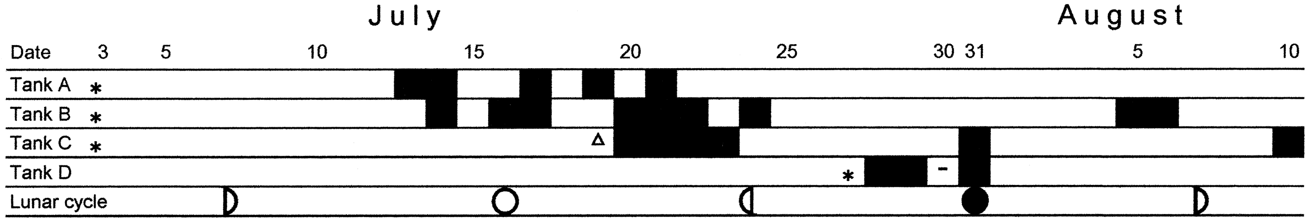

Fig. 4. Dates of spawning in tanks during the spawning period in 2000. Solid squares indicate the spawning days. The lunar cycle is indicated by a new moon (solid circle), full moon (open circle), first-quarter moon (open right half), and last-quarter moon (open left half). The identifying names of the tanks are described in Materials and Methods. Asterisks indicate the first day on which animals were kept in each tank. The open triangle indicates the first day on which the black cloth was used to cover tank C at night. The "–" on July 30 indicates no record due to trouble with the video camera.

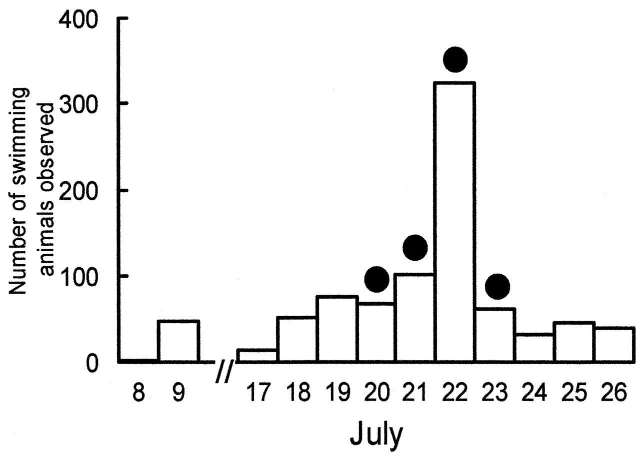

Fig. 5. The number of swimming amphioxus observed per day from July 8 to July 26 in tank C. Solid circles indicate spawning days.

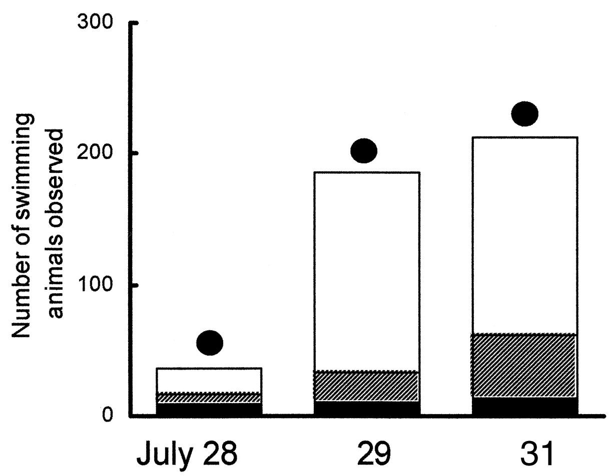

Fig. 6. The numbers of amphioxus that swam up less than 50 cm (solid column), between 50 cm and 90 cm (shaded column), and to the surface (open column), respectively, on July 28, 29, and 31 in tank D. Solid circles indicate the spawning days.

DISCUSSION

We report here, to our knowledge for the first time, the spontaneous spawning of amphioxus under the captive condition in Japan. We were not successful in inducing spawning in captive amphioxus in the preceding four breeding seasons from 1996 to 1999, although we kept animals under similar conditions. In 2000, we improved the feeding and environmental conditions. More specifically, the tanks were kept in a room isolated from other laboratory areas in order to prevent amphioxus from the stress such as vibration of the tank caused by human walking. These improvements may help to explain the success this time. In addition, our result in tank C suggests that strong light suppresses spawning, because spawning in tank C started after covering the side of the tank with a black shading sheet during daytime. In China, Wu *et al.* (1994) succeeded in inducing captive spawning in the same species of amphioxus. They used a tank 47 cm deep and the tank water temperature was 21°C or higher. In addition, his group reported that larvae obtained in the laboratory became adults by the next breeding season and spawned in the tank in his laboratory (Zhang *et al.*, 2001). Our next goal will be the continuous breeding of this species in captivity in Japan.

Spawning occurred at irregular durations and various intervals. In some cases, it lasted for several days, but in other cases, it stopped next night. We may consider that timing of the spawning partly depends on the maturational condition of the gonads. However, a synchronization mechanism would exist at the same time to explain the variation in the spawning duration and interval. Wickstead (1975) reported finding no spawning migration or aggregation in amphioxus. If there spawning migration or aggregation occurred in *B. belcheri*, only mature animals could be expected to be found at a station. However, animals at various stages of gonad maturation were collected at every station in our study. These findings support those in a previous report by Wickstead (1975). However, we found that the gonad maturational stages differed not only among stations but also between the two sea areas, Deyama and Takamatsu. Differences among stations would indicate that gonad maturation does not synchronize so strictly, and that mobility of animals lowers as they settle within a particular station. Furthermore, the relation of differences between environment and gonad maturation among stations should be studied in detail.

The difference in distribution of gonad maturational stages between Deyama and Takamatsu may most likely be explained by the presence of post-spawning animals in Takamatsu and their absence in Deyama. The sum of proportions of mature and post-spawning animals in Takamatsu is almost equivalent to the proportion of the mature animals found in Deyama. These facts indicate that some terminated spawning in Takamatsu, whereas none had done so yet in Deyama, although gonad maturation was roughly synchronized in these two places. Accordingly, the collective weight of these results suggests that *B. belcheri* is a settled species.

It is not yet known whether the gonad of amphioxus regresses completely after spawning and does not show recrudescence during the same breeding season or whether it recovers soon after spawning for purposes of re-spawning in the same breeding season (Chin, 1941; Stokes, 1996). However, the absence of the post-spawning animals in the Deyama population and their presence in the Takamatsu population may support the former possibility. We sorted out the post-spawning animals and kept them in a separate tank for a month or so, and subsequently found that these animals did not show gonad recrudescence.

We found that males were more numerous than females in Deyama and Takamatsu populations of *B. belcheri*. The sex ratio for the total of both Deyama and Takamatsu was 1.21 (males/females). A similar male-biased sex ratio; average is 1.32 : 1 was previously reported for the Xiamen population of the same species which was monthly collected through the year (Fang and Qi, 1990; the ratio was calculated from the number of animals described in the text.). In contrast, Stokes and Holland (1996) reported a 1 : 1 sex ratio in *B. floridae*.

Amphioxus is a benthic animal occupying shallow waters less than 70 m in depth of intertidal regions. In such a habitat, amphioxus is exposed to many environmental variables, including temperature, light, salinity, and tidal current. In the present study, animals were kept in tanks of which tops were not covered and under the natural daily light condition. We found no correlation between the spawning time and the lunar cycle. Similarly, Stokes (1996) reported no correlation between spawning and tidal change or lunar cycle. Photoperiod, however, has been reported to influence gonad development, maturation, and spawning in amphioxus (Fang *et al*., 1989, 1992). According to Fang's report, a long photoperiod (16L: 8D) is unfavorable to the spawning of amphioxus, whereas a short photoperiod (8L: 16D) is favorable to spawning and reproductive activity. Artificial illumination for two hours after sunset shifted the spawning time, whereas darkening for two hours before sunset had no effect (Watanabe *et al*., 2000). These studies unanimously showed that light suppresses spawning, although it is probably not a trigger of the spawning. Our results suggest that intensity of light during daytime is a suppression factor of spawning in amphioxus. However, further experimental study is required to confirm this suggestion.

In our laboratory observation, amphioxus swam up 90 cm to water surface and released gametes on the surface. According to the review of Wickstead (1975), adult amphioxus often emerge from the bottom at about sunset, swim around freely near the bottom and spawn although details of the study were not described. Our observations of spawning behavior clearly differed from their observations. Differences in environmental conditions including the depth of tank water may be the reason of the disagreement.

In our case, a high density of amphioxus individuals in the wild population might ensure mixing of the gametes in water such as Webb (1958) reported. Spawning in water would increase the possibility of propagation of the species. In our recording equipments, we could not discriminate between the male and female at the time of spawning. However, in our observation, synchronization of swimming by more than one animal did not appear through the observing period. It was confirmed that both sexes spawned, given that developing embryos were found in the tank water at the time the water was changed the next morning. Fertilization might occur properly in the tank even though male and female freely released their gametes there. However, our preliminary observation showed that when males and females separately put in the tank with partition wall, they did not swim, although more precise study is needed to confirm this observation. The male-female interaction or communication necessary for synchronization of spawning might occur before or after animals emerged from the sand at the bottom of the tanks. Further study is needed to elucidate the mechanisms underlying male-female interaction at the time of spawning.

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