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Biology of the Amphioxus, *Branchiostoma belcheri* in the Ariake Sea, Japan I. Population Structure and Growth

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ABSTRACT—We investigated the population structure and growth of the amphioxus *Branchiostoma belcheri* for four years in the southern Ariake Sea, Japan. We counted 62-66 myotomes and 251-310 dorsal fin-ray chambers, and these results support that this species is an intermediate form of *B. belcheri* and its subspecies *B. belcheri tsingtauense*. The ratio of females to males was 1:1.12. Males were more numerous than females among small individuals (< 40 mm body length), but we found no significant differences among large animals (≥ 50 mm body length). Spawning occurred from mid June to early July. Groups of newly settled young appeared from January to June of their second year. We observed a large fluctuation between years in the numbers of newly settled young. The estimated size of one-year-old individuals was 19.4 mm in body length; within the next 12 months, they reached 32.1 mm. Three- and four-year-old individuals measured 38.6 mm and 45.8 mm, respectively. Few grew beyond 60 mm; the largest specimen collected was a 64 mm male.

Key words: amphioxus, Ariake Sea, *Branchiostoma belcheri*, growth, population

INTRODUCTION

Nakagawa (1897) first reported the occurrence of amphioxus near the Amakusa Islands. He obtained specimens off Goshonoura Island, south of Amakusa-Kamishima Island (Fig. 1) and he subsequently described the reproductive period and morphology of the animal. The body length of the largest of 126 measured individuals was 54 mm, which the smallest was 10.5 mm. Nakagawa (1897) also examined the number of myotomes in 58 specimens; 35 had 64 segments, and the average number of myotomes was 64.1 (Table 1). These morphological characteristics did not match any of the nine species described by Andrews (1895).

The occurrence of the amphioxus in the Ariake Sea on the opposite side of Amakusa-Kamishima Island was first reported by Azuma (1904), who found a small fish-like animals that has a local name of “itachi-uo” in a sandy tidal flat in the northern Ariake Sea were an amphioxus. Note that a formal English name has not been established for the Ariake Sea; thus, the bay has been variously termed Ariake Sound, Ariake-kai, or Ariake Bay. The southern half of the bay has also been given different name, Shimabara Bay. However, because this name is never used by bay-area residents, we have adopted the expression used by Nishikawa (1981).

Ohshima (1927) mentioned the occurrence of this animal in another tidal flat located in the northern Ariake Sea. Oyama and Yoshii (1940) carried out further observations. They obtained specimens from a tidal flat on the southern coast of Saga Prefecture, which they called “Aratsu” or “Gandotsu”, and reported that the reproductive period lasted from the end of May to early June. They suggested that the animals belonged to the species *Branchiostoma belcheri*. A very extensive study of Japanese amphioxus was conducted by Nishikawa (1981), who examined preserved specimens from universities, museums or private collections, including samples obtained from the Ariake Sea and the Amakusa Islands. He found that specimens from the Ariake Sea had 64-65 myotomes, while specimens obtained from other Japanese localities had 66-67. He therefore concluded that amphioxus in Japanese waters other than the Ariake Sea and the Amakusa Islands belonged to *B. belcheri* var. *tsingtauense*, and that animals found in the Ariake Sea and the Amakusa Islands were an intermediate form between this variety and *B. belcheri*. Yamaguchi and Kikuchi (1985) described the distribution of amphioxus in the Ariake Sea and the Amakusa Islands. They showed that *Branchiostoma* occurs in the central area of the Ariake Sea and four locations surrounding the Amakusa Islands.

Several authors have conducted biological and ecological studies of amphioxus. Chin (1941) studied *B. belcheri* in

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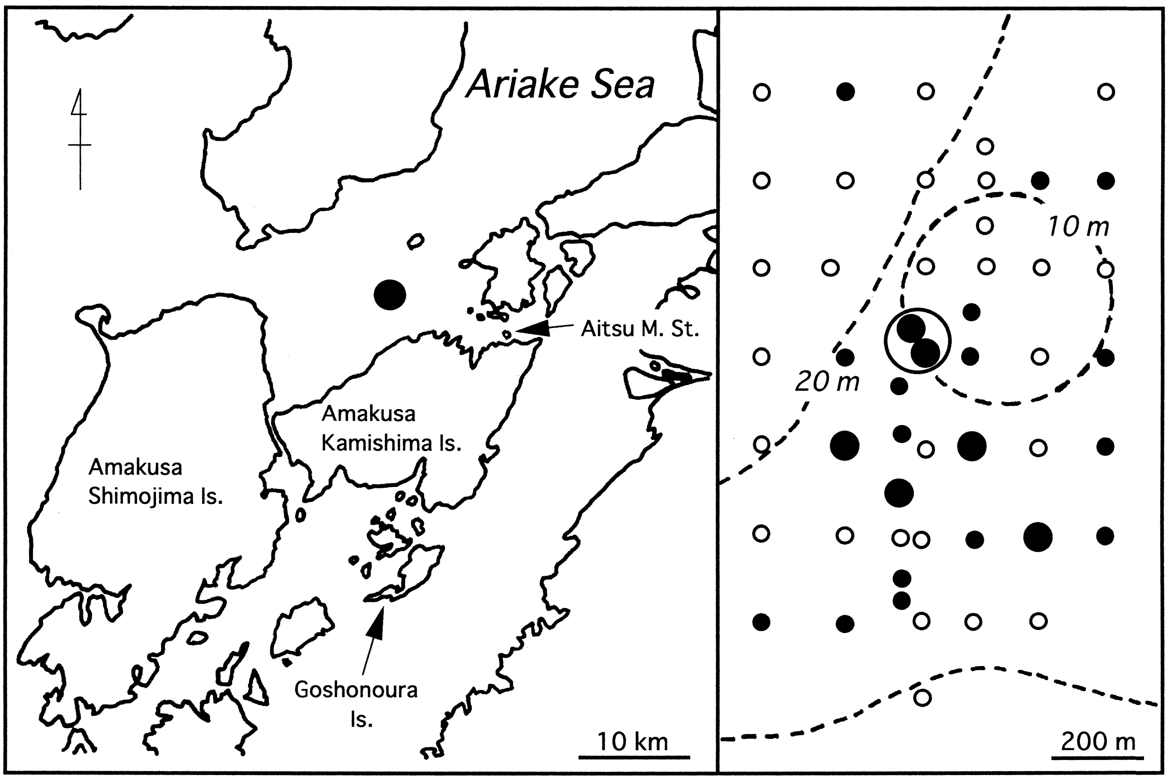

Fig. 1. Left: Map showing the location of the collection site (filled circle) in the Ariake Sea, Japan. Right: Map showing the distribution of the amphioxus *Branchiostama belcheri* in the study area. Solid circle indicates the site where specimens were collected by dredging. Small and large filled circles show sites where one to four, and five to ten specimens were collected using the Smith-McIntyre grab-method (with a sampling area of 22×22 cm), respectively. Open circles show the sites where no animals were obtained.

Table 1. Morphological characteristics (mean±SD, range) of *B. belcheri* in the Ariake Sea and the results reported by Nakagawa (1897). 1, Number of dosal fin-ray chambers; 2, Number of preanal fin-ray chambers; 3, Number of myotomes from the anterior end to the atriopore; 4, Number of myotomes from the atriopore to the anus; 5, Number of myotomes from the posterior end to the anus; 6, Total number of myotomes; 7, Average body length (mm).

		1	2	3	4	5	6	7
Present study								
Males (n=53)	Right side	282.5±12.5 (251–307)	55.4±4.9 (46–66)	36.2±0.8 (35–38)	17.2±0.5 (16–18)	10.4±0.7 (9–12)	63.8±0.8 (62–66)	43.2±6.0 (30–56)
	Left side			36.4±0.6 (35–38)	17.2±0.5 (16–18)	10.3±0.6 (9–12)	63.9±0.8 (62–66)	
Females (n=51)	Right side	282.4±10.5 (262–310)	55.4±3.5 (48–62)	36.1±0.8 (35–38)	17.3±0.5 (16–18)	10.2±0.7 (9–12)	63.6±0.9 (62–66)	41.3±7.0 (25–53)
	Left side			36.4±0.8 (35–38)	17.2±0.4 (17–18)	10.1±0.5 (9–11)	63.8±0.8 (62–66)	
Nakagawa (1897))	(n=58)	no data	no data	36.5	17	10.6	64.1	38.6 (10.5–54)

Amoy, China, and Xianhan *et al.* (1995) investigated the life history of *B. belcheri tsingtauense* in Quingdao. Courtney (1975) described the age structures of *B. lanceolatum* in the North Sea, Stokes and Holland (1996a) examined the life history of *B. floridae*, and Webb (1958) studied an African species, *B. nigeriense*. However, no detailed studies dealing with the change of population structure for a longer period have been conducted of Japanese amphioxus.

Nishikawa (1981) reported that suitable habitats for amphioxus in Japan had been destroyed. Two localities of the amphioxus, near Uryu Island in the Seto Inland Sea and Oshima Island in Ise Bay, had been known as a good habitat of the animal and were designated to be natural monuments. However, the animal disappeared from there as a result of the change in the bottom sediment (Nishikawa and Mizuoka , 1990; Nishikawa, 1995).

We discovered a site ca. 3.5 km from Amakusa-Kamishima Island (Fig. 1) that is still inhabited by amphioxus, although the environmental conditions of this habitat continue to deteriorate. For the past ten years, one or two groups of vessels have daily collected sand that is deposited on the ocean floor. In areas where they have completed excavations, the floor is three to ten meters lower than in surrounding areas and is covered with boulders or cobbles, rendering it inhospitable to amphioxus. Another habitat, located ca. 2 km east of the study area, has been completely destroyed. Sand that siphoned up from the floor by these vessels is washed, and small stream of seawater containing minute particulate matter run back into the sea. These particles are carried several kilometers from the vessels by tidal currents and dispersed, causing changes in the sediment texture of the sea floor.

Nishikawa (1995) described the Japanese amphioxus *B. belcheri* as endangered. He emphasized that the Ariake Sea population was of particular importance and that the reason why a specific form of the species occurs in this area warranted investigation. We believe that it is very important to study the biology of an animal before the complete destruction of its habitat; thus, we began collecting amphioxus by dredging in January 1999 and continued until September 2002.

MATERIALS AND METHODS

Grab-method of sampling

To investigate the distribution of the amphioxus within the habitat, we collected a total of 55 samples between 12 April and 12 September 2002 using the Smith-McIntyre grab-method with a sampling area of 22×22 cm (Fig. 1, right).

Collection of amphioxus

We chose one site within the habitat, from which specimens were collected by dredging between 27 January 1999 and 12 September 2002 (Fig. 1, right, solid circle). A 2-cm mesh net (60 cm deep) was attached to a stainless steel frame (40×80 cm) and pulled by a ship (15.4 m long, 9.5 tons). Water depth at the site was about 10 m at maximum low tide. However, tidal currents flowing at a maximum speed of 2 knots prevented us from dredging at exactly

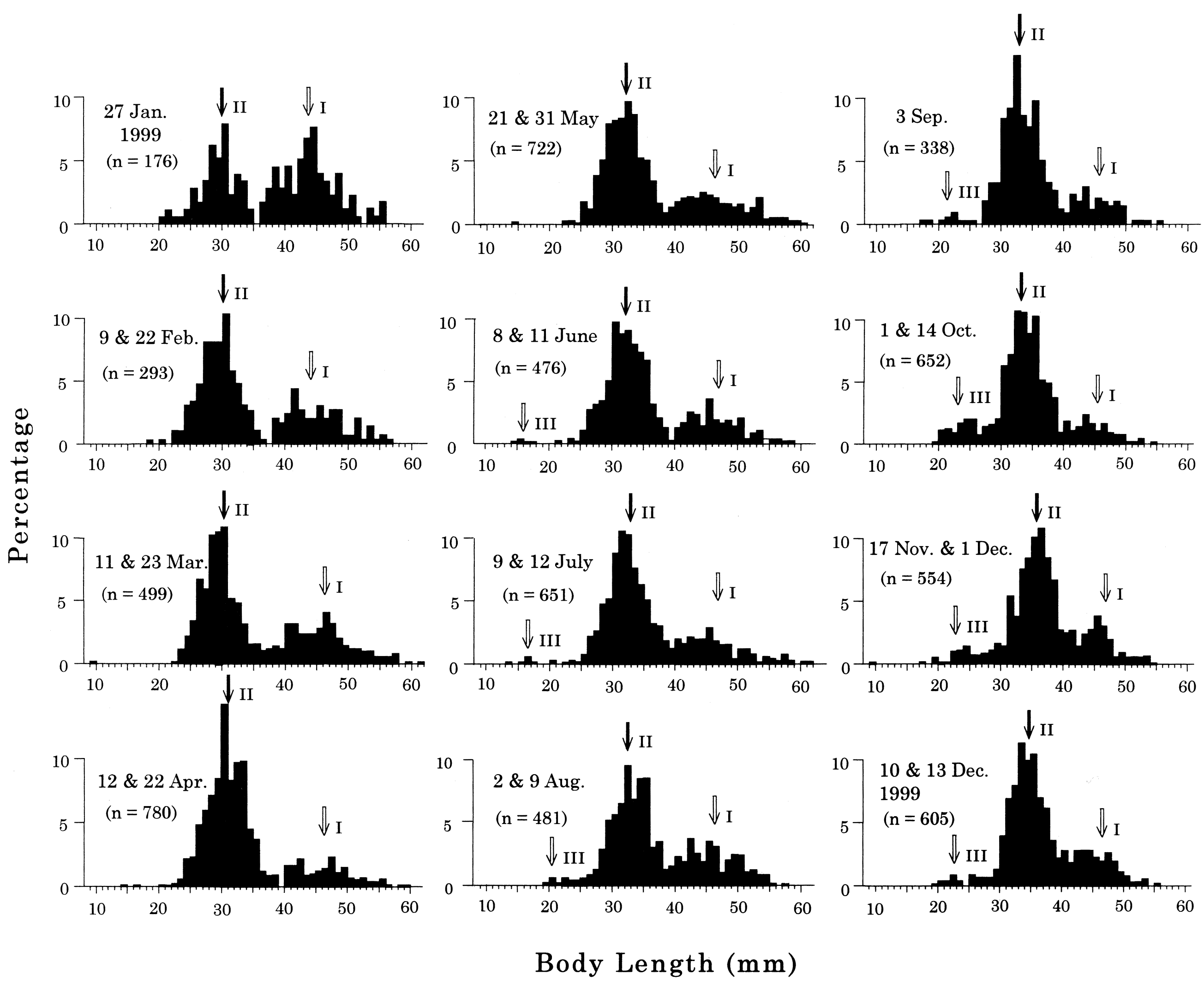

Fig. 2. Size-frequency distribution of *B. belcheri* collected in 1999. Arrows with Roman numerals indicate the average body length of the different age groups. Each age group was distinguished by plotting cumulative size-frequencies on a probit scale.

the same spot each time. Collected sand was examined to determine whether it contained amphioxus. Sand that contained too few animals was discarded and dredging was repeated. We typically dredged three to six times, and collected 100 to 150 liters of sand, which was then taken to the laboratory to remove all of the specimens that it contained. After careful examination of the sand, we placed a subsample (2–3 L) in a bucket to which we added fresh water. The sand was stirred by hand, and the water was discarded through a small net. Half-paralyzed, motionless amphioxus were collected from the net and placed in small jars filled with seawater. This process was repeated until no more animals were found. Most amphioxus survived the treatment, and collected animals were kept alive until we had measured body length and gonad size. Animals that were already dead prior to the measurements were kept in a refrigerator, and after measurement all specimens were preserved in 3% seawater formalin.

In addition, specimens were collected by dredging in the collection site on 1 May 2001,and in a location ca. 100 m apart from the collection site on 16 May 2001, in order to compare the size composition of animals. Collected sand was analyzed of particle size. A sample of ca. 500 g of sand was passed a series of sieves (mesh size: 1.0, 0.5, 0.25, 0.125 and 0.063 mm), and the different size fractions weighed after drying at 80°C for three days. Percentages relative to the whole dry weight were calculated.

Measurement of the body length and gonad size

Each live specimen was placed in a thin, transparent plastic petri dish 5–7 cm in diameter, to which clean seawater was added. The dish was then placed on 1-mm mesh plastic paper, and body length (BL, from the tip of the snout to the end of the tail) was measured to the nearest 0.5 mm. Three of the longest gonads were measured and the average of these three values was used to describe gonad length (see Part II).

Sexing

Sex determination is possible when amphioxus have developed gonads. Well-developed ovaries are yellow and oocytes are obvious under a stereomicroscope. Testes are whitish, and no granules are visible inside. However, some females possessed whitish rather than yellowish ovaries, which made it unreliable to determine sex only by color. We therefore identified sex under a stereomicroscope, based on the presence or absence of oocytes.

Counting of myotomes

We used only freshly killed individuals, collected on 1 May 2001 and 20 March 2001. Each live animal was placed in 2% seawater formalin and removed after one minute. After measuring body length, the number of myotomes was examined under a stereomicroscope. In addition to myotomes, we also examined the number

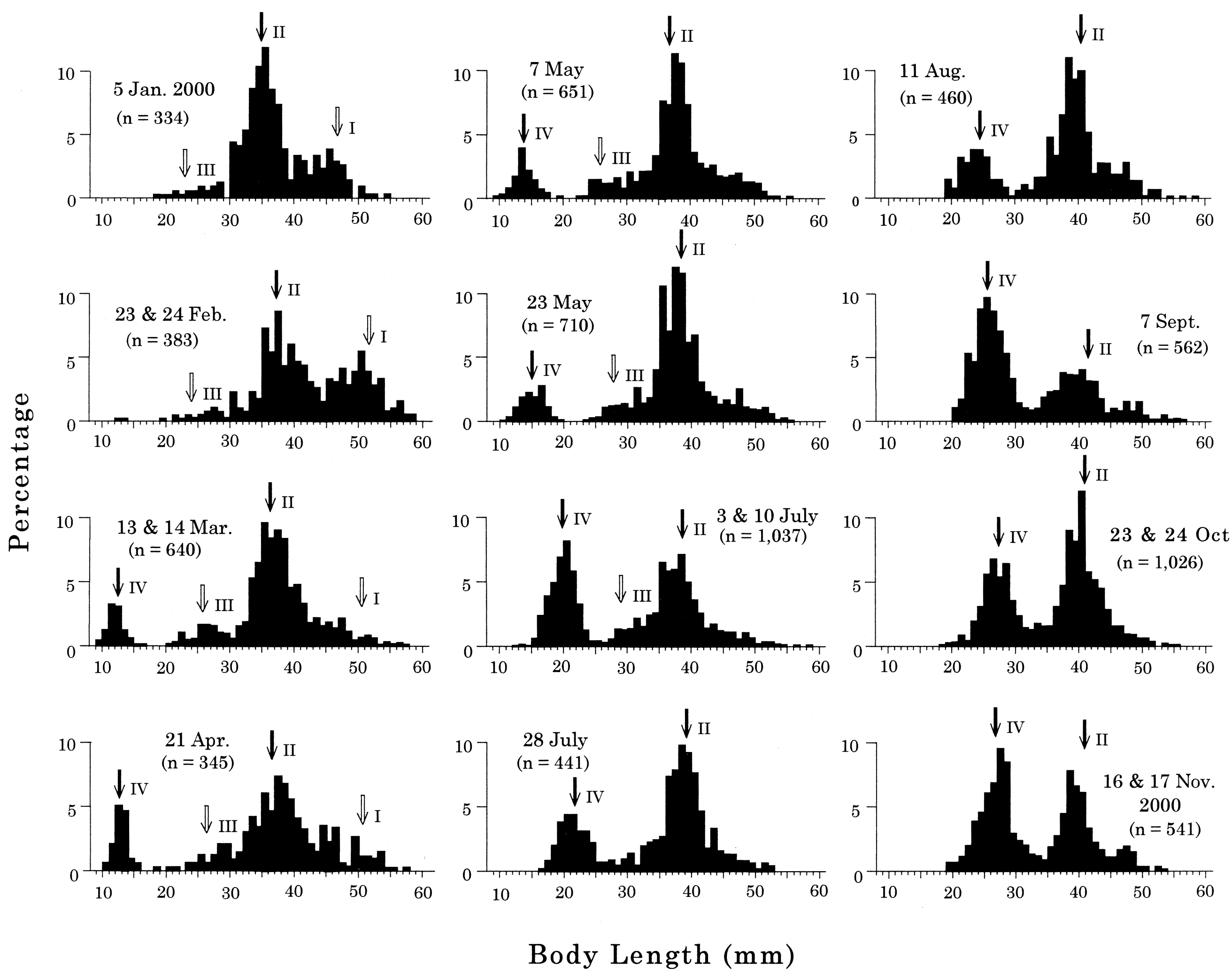

Fig. 3. Size-frequency distribution of *B. belcheri* collected in 2000. Details as in legend to Fig. 2.

of fin-ray chambers.

RESULTS

Distribution of amphioxus

Henmi *et al.* (2000) presented a map illustrating the present distribution of amphioxus in the southern Ariake Sea. This map was based on unpublished data of benthos survey conducted by Nagasaki University in 1997. The map indicates that the habitat from which we obtained our samples is the nearest habitat to the Aitsu Marine Station. Our collection site was located ca. 3.5 km north of Akasaki, in the Amakusa-Kamishima Island. The sea floor near the site was about 10 m higher than in surround areas and was covered with broken shells and sand. Amphioxus were patchily distributed in this habitat and seemed to be concentrated in a relatively small area. The density obtained by the Smith-McIntyre grab-method at the site was 139.5±68.3 (mean±SD, n=4) individuals/m^2.

Morphological characteristics

We obtained a total of 23,051 specimens in 63 collections between 27 January 1999 and 9 January 2002. The largest individual (male), collected on 1 July 1999, had 64 mm BL, and the smallest individual, collected on 5 January 2001, had 7 mm BL. Table 1 summarized the results of the morphological examination. We counted an average of 63.8 and 63.7 myotomes, 282.5 and 282.4 dorsal fin-ray chambers in males and females, respectively. Thus, the differences between sexes did not detected in these characteristics.

Population structure and growth

Fig. 2 shows the size-frequency distributions of samples collected in 1999. Samples obtained within a two-week period were combined and are presented as a single sample. Each histogram shows several peaks, and the histogram of 27 January 1999 indicates two large groups. Each sample consisted of several age groups, but similarities between some of them made it impossible to differentiate all age groups. For convenience, we considered the first sample, of 27 January 1999, to consist of only two age groups (Groups I and II) that were normally distributed. Small groups that appeared in the histogram were included in one

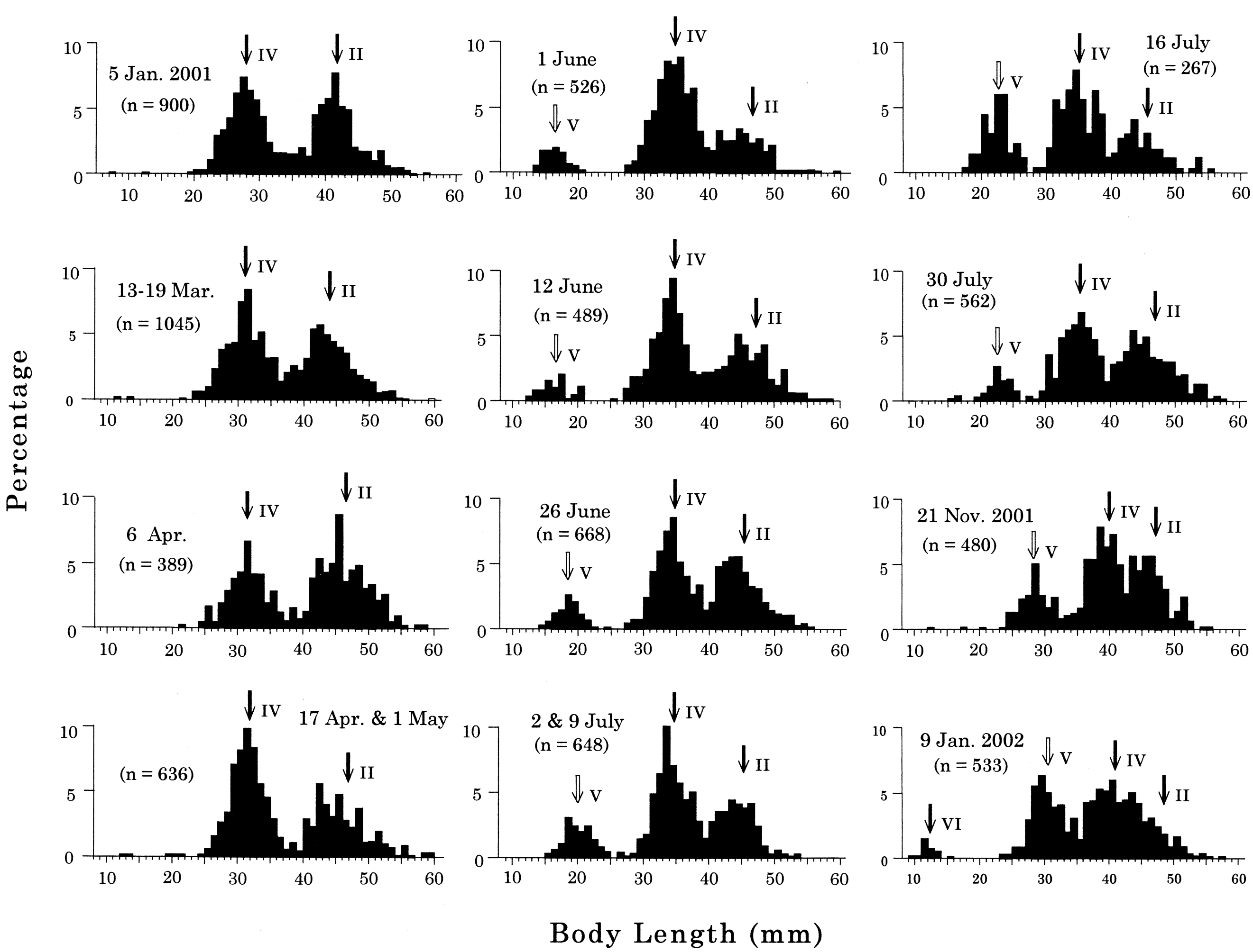

Fig. 4. Size-frequency distribution of *B. belcheri* collected in 2001 and on 9 January 2002. Details as in legend to Fig. 2.

of the two major groups. By so doing, we were able to trace the two groups through subsequent histograms.

The group that peaked at ca. 45 mm BL (Group I, indicated by an open arrow) appeared continuously in subsequent histograms; however, the size of the group fluctuated greatly. Group I occupied only 18.3% of the sample from 12 and 22 April (143/780), but 59.1% of the sample from 27 January (104/176). The percentage of Group II, which averaged ca. 30 mm BL (indicated by a solid arrow) in the histogram of 27 January, increased in subsequent histograms. In 1999, very few young individuals settled; one 9-mm BL individual was included in the sample obtained between 11 and 23 March. The April to July samples contained one to five individuals between 13 and 18 mm BL, which constituted < 0.17% of the samples. Although few in number, these young individuals constituted a separated age group (Group III), which is indicated in Fig. 2 by an open arrow. This group (III) increased to 7% of the total (46/652) in the histogram for 1 and 14 October, but remained a small percentage in most histograms.

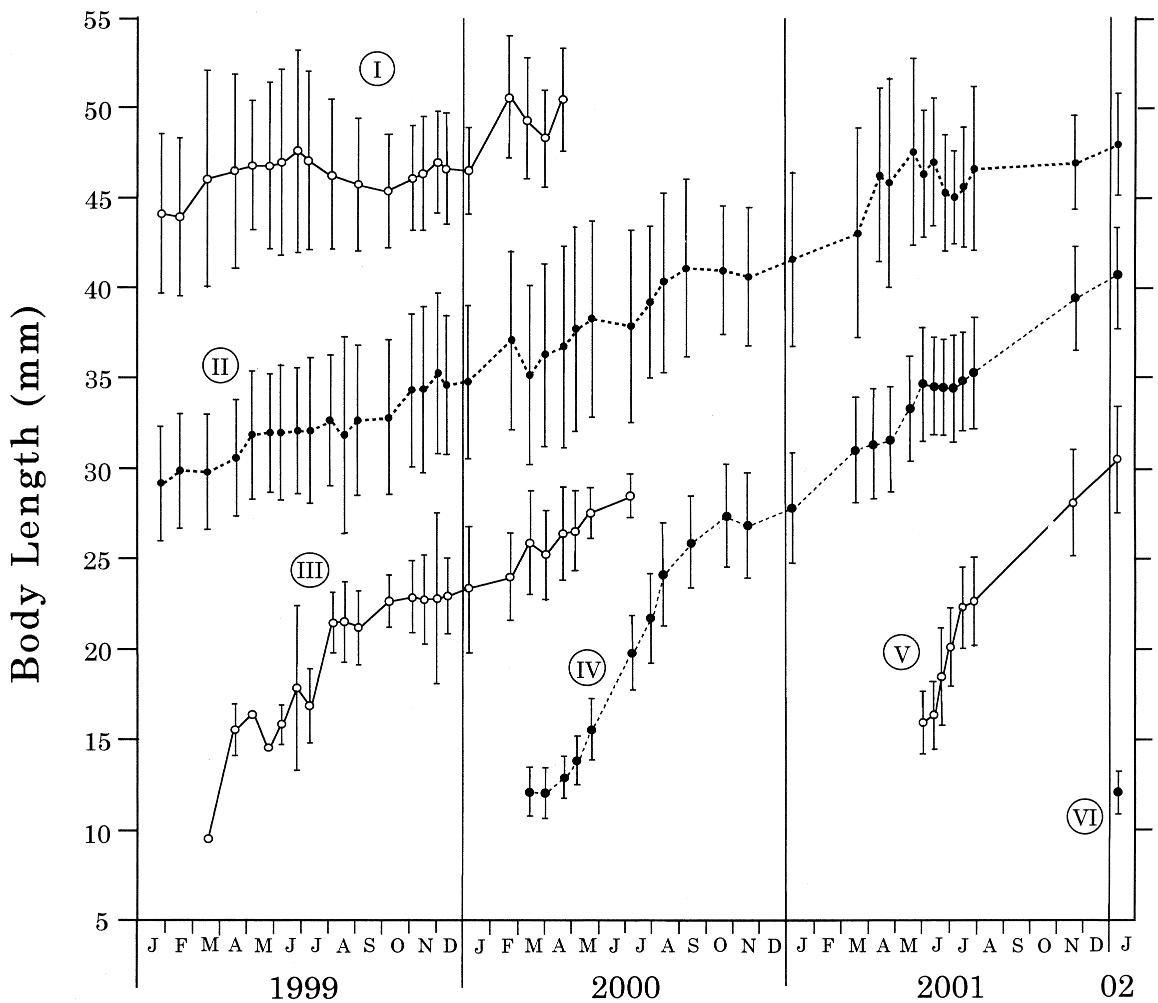

Fig. 5. Growth curves of *B. belcheri*. Circled Roman numerals indicate age groups. The average body length of each age group is connected, and the vertical bars represent SD.

Table 2. Average body length of each age group of *B. belcheri* in the Ariake Sea, Japan.

Age (year)	Average body length (mm)	Age group
1	19.4	Group III, IV and V (July 1999–2001)
1.5	29.3	Group IV and V (January 2001, 2002)
2	32.1	Group II (July 1999)
3	38.6	Group II (July 2000)
4	45.8	Group II (July 2001)
5	47.1	Group I (July 1999)

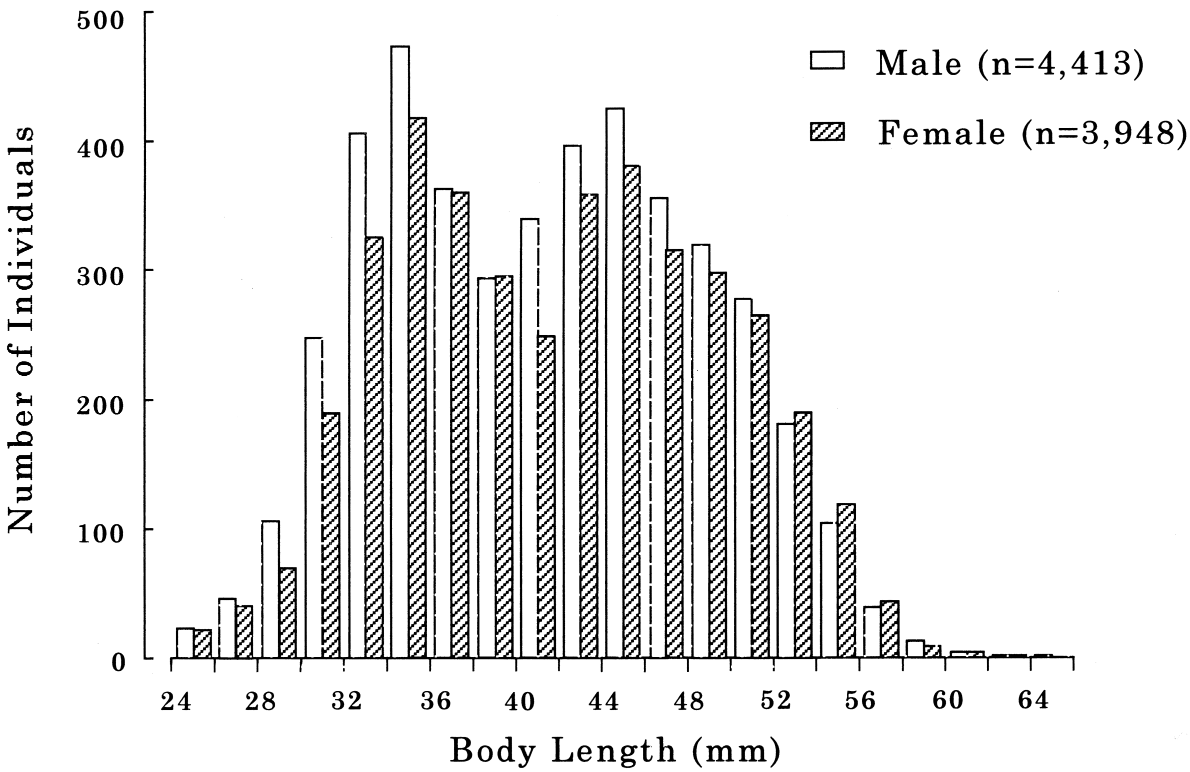

Fig. 6. Size-frequency distributions of male (open bars) and female (hatched bars) *B. belcheri*. Only specimens obtained from mid May to mid July in 1999–2002 were counted.

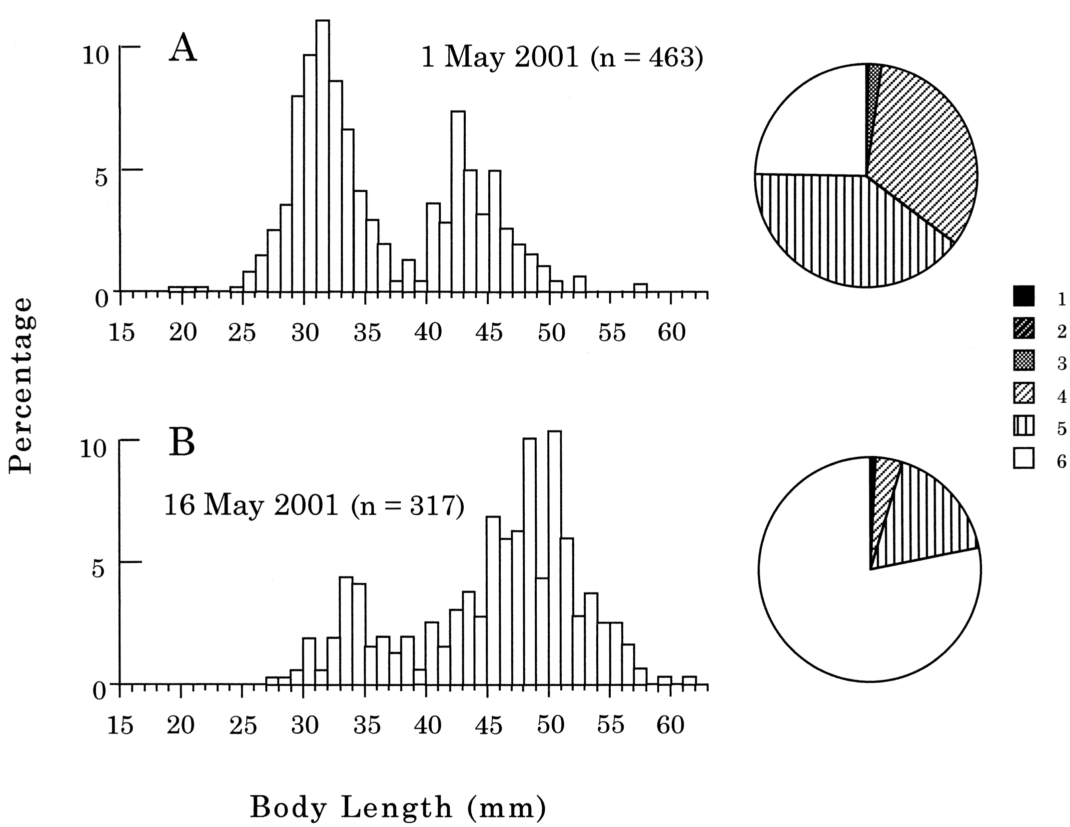

Fig. 7. Size-frequency distributions of *B. belcheri* obtained on 1 May 2001 (A) and 16 May 2001 (B). Specimens in (A) were obtained at the collection site, while specimens in (B) were collected at a location 100 m away. Sediment composition at each site is shown in the pie graphs. Granule sizes: 1, < 0.063 mm; 2, 0.063–0.125 mm; 3, 0.125–0.25 mm; 4, 0.25–0.5 mm; 5, 0.5–1.0 mm; 6, ≥ 1.0 mm.

Fig. 3 shows the histograms for samples obtained in 2000. Group II dominated, while Group I lost its identity and was incorporated into Group II in the sample of 7 May. Group III became less apparent and could not be distinguished after 28 July. The incidence of newly settled individuals (Group IV) differed markedly from 1999. In the samples from 13 and 14 March, a small but distinct group averaging 12.2 mm BL suddenly appeared, whose numbers subsequently increased and surpassed Group II in the sample from 7 September.

Fig. 4 shows the samples collected in 2001 and on 9 January 2002. The size of Group II gradually decreased, but the group remained apparent in all histograms. The incidence of newly settled young individuals was very different from 1999 and 2000. On 5 January, we caught the smallest individual (7 mm BL) recorded in this study. However, few young individuals appeared in the samples from March to May, but they suddenly appeared as a distinct group (Group V) in the June samples. The sample from 9 January 2002 also contained a group of young individuals (Group VI); in the previous three years, no such early appearance of young individuals had occurred.

From the changes in the average values of each group, it is possible to estimate the growth of amphioxus; Fig. 5 summarizes these results. Group II was evident throughout the study. Spawning occurs from mid June to early July (see Part II). Therefore, the smallest individuals that appeared in July were about 12 months old. The average BL of individuals in Group III, IV and V in July 1999–2001 were 17.2, 20.8, and 20.1 mm, respectively; the average of these three values is 19.4 mm, which we will use to describe the size of a one-year-old animals. In January of the following year, these individuals were 1.5 years old. The average BL of the three groups were then 24.0, 27.8, and 30.7 mm; of these three values, Group III was the least reliable because of few individuals (15/334). Therefore, we discarded the mean length of Group III. The average BL of Group IV and V in January 2001, 2002 were 29.3 mm. Since this is almost the same as the body length of Group II in January 1999 (29.2 mm), it is reasonable to assume that Group II in January 1999 was comprised of 1.5-year-old individuals.

From the changes in the size of Group II specimens, it is possible to infer further growth patterns. In July 1999, individuals in Group II were two years old and averaged 32.1

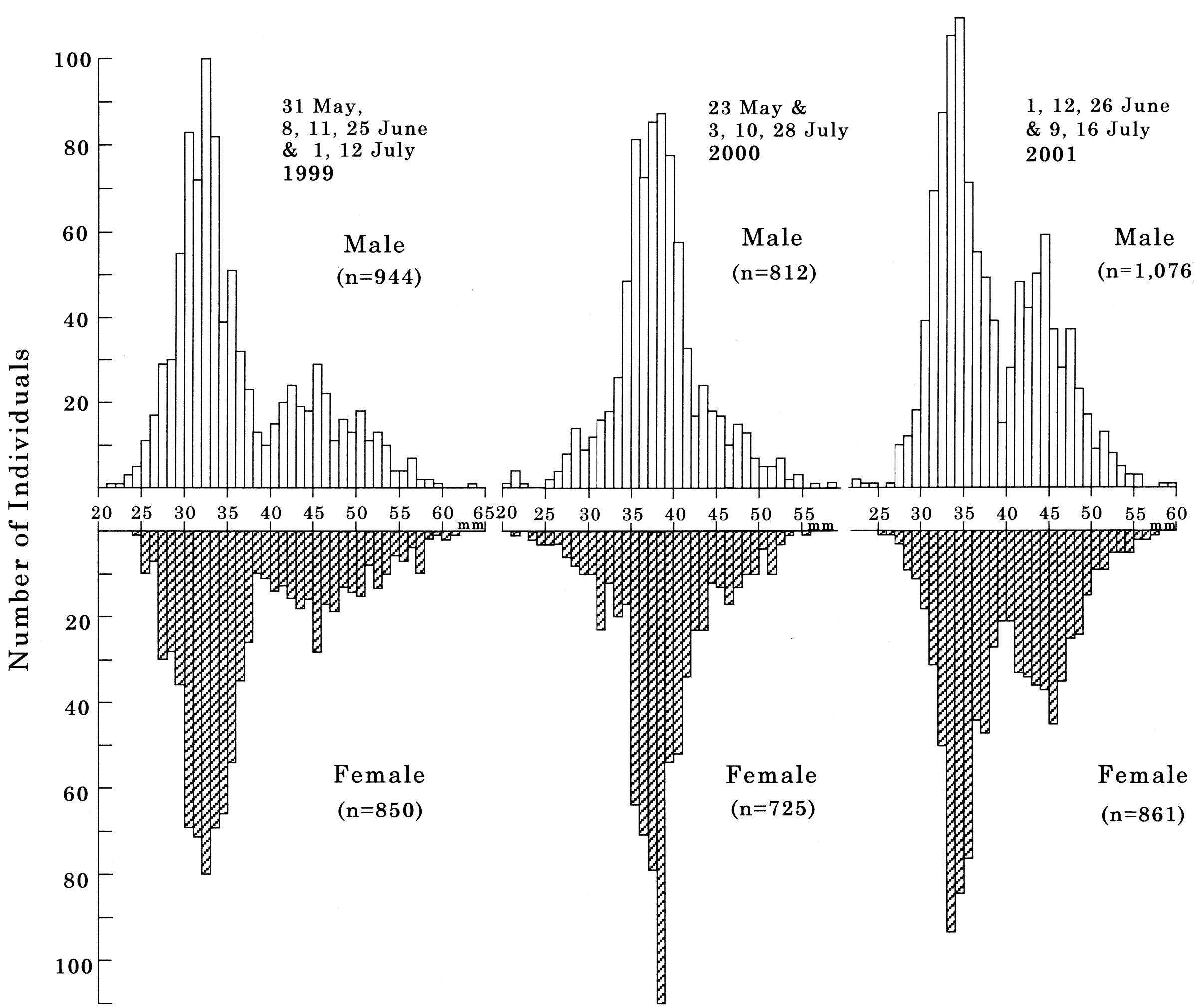

Fig. 8. Size-frequency distributions male (above) and female (below) *B. belcheri* obtained between late May and early July of 1999–2001.

mm BL, whereas in July 2000, at three years of age, they averaged 38.6 mm BL; their average BL increased to 41.9 mm by January 2001, and 45.8 mm by July 2001. Group I measured 44.1 and 47.1 mm BL in January and July of 1999, respectively, which was larger than Group II in 2001. Therefore, it is likely that individuals in Group I were not two but three years older than those in Group II, and that they were therefore 4.5 years old in January 1999 and five years old in July 1999. These assumptions are summarized in Table 2.

Fig. 6 shows a size-frequency histogram of the two sexes. During the period from September to December it was very difficult or impossible to determine sex because gonads were transparent and very small. After January, gonad size gradually increased and reached the maximum in June. Unsexable animals composed < 1% of the samples obtained from mid May to mid July. Only data based on samples obtained during this period are presented in Fig. 6, which includes not only the specimens obtained between 1999 and 2001, but also samples collected during the reproductive period of 2002. The size composition was not significantly different between sexes (P>0.05, d=0.026, Kolmogorov-Smirnov two-sample test), but total F:M ratio was 3,948:4,413=1:1.12, which differs significantly from an expected sex ratio of 1:1 (χ^2 = 25.86, P<0.01, χ^2-test). However, this ratio changed with body size. For smaller individuals (< 40 mm BL) the ratio was biased towards males (1,717:1,957=1:1.14; χ^2 = 15.68, P<0.01, χ^2-test), whereas the ratio was reversed in individuals ≥ 50 mm BL (632:621=1:0.98), which was not significantly different from the expected 1:1 ratio (χ^2 = 0.097, P>0.05, χ^2-test).

Fig. 7 shows size frequency histograms and sediment texture of two samples obtained in May 2001. The two samples were collected within only 15 days, but the size composition was significantly different (P<0.001, d=0.515, Kolmogorov-Smirnov two-sample test). The sample in Fig. 7 (B) was collected on 16 May from a location covered mostly with broken shells that was only ca. 100 m from the collection site, and contained mostly larger individuals. The percentage of individuals ≥ 45 mm BL was 65%, while in the sample obtained at the collection site on 1 May (A) it was only 13%.

Fig. 8 shows the frequency distributions of the specimens obtained in June and July over three years. The number of specimens differed between the two sexes; however, size frequency patterns were symmetrical and the size composition was not significantly different between sexes in each year (each P>0.05, d=0.057, 0.051 and 0.060 in 1999, 2000 and 2001, respectively, Kolmogorov-Smirnov two-sample test).

DISCUSSION

Morphological characteristics

In our study of *B. belcheri* in the Ariake Sea, we counted 62-66 myotomes, and 251-310 dorsal fin-ray chambers. Andrews (1895) described the morphological features of nine species, including *B. belcheri*, but his descriptions do not correspond to specimens found in the Ariake Sea. These results support Nishikawa's (1981) conclusion that the species is an intermediate form of *B. belcheri* and its subspecies *B. belcheri tsingtauense*.

Population structure and growth

In our study, we assumed that the average size of one, two and three-year-olds was 19.4, 32.1 and 38.6 mm BL, respectively. And that of four and five-years-olds was assumed to 45.8 and 47.1 mm BL, respectively. The total number of individuals ≥ 50 mm BL among the 23,051 specimens examined was 1,054 (4.6%); these individuals had likely survived more than five years. Six individuals exceeded 60 mm BL and such large individuals may be eight years old or more. Chin (1941) studied the population structure and growth of *B. belcheri* in Amoy, China. He classified individuals by age and showed that 8.8-mm BL individuals, which settled between 14 June and 13 July 1931, grew to 17.7 mm BL by January 1932 and 38.4 mm BL by December 1932. The same individuals measured 41.1 mm BL in January 1933 (1.5 years) and grew to 49.2 mm BL by December (2.5 years). Fourth-year amphioxus, aged 2.5–3.5 years, measured ca. 53 mm BL. The largest individual was 57 mm BL. These values indicate that the annual growth rate is faster in Amoy than in the Ariake Sea.

Xianhan *et al.* (1995) reported that growth of *B. belcheri tsingtauense* in Quindao was faster in summer and autumn than in winter and spring, whereas we found no seasonal differences in growth in our study. In Quindao, the lowest water temperature was 2.2°C, whereas the lowest water temperature recorded in our study 11.8°C. Chin (1941) found no clear termination of growth in winter in *B. belcheri* at Amoy, where the lowest water temperature was 12.0°C. Winter water temperatures in the Ariake Sea and Amoy are far higher compared to Quindao; therefore, it is likely that growth of amphioxus continues during winter.

Fluctuations in size-class distributions within each sample prevented us from obtaining more reliable growth curves for amphioxus. The fluctuation reflects the uneven of animals in the habitat. As shown in Fig. 1 (right), amphioxus occurred patchily, not uniformly. The area of the collection site was covered with a mixture of broken shells and sand, but composition was not uniform. We aimed to collect specimens from exactly the same spot, but tidal currents prevented us from doing so. Where sediment consisted mostly of broken shells, the ratio of larger individuals increased, but it decreased in areas covered with fine sand (Fig. 7).

Local differences in sediment texture result in a patchy distribution of amphioxus. Kubokawa *et al*. (1998) reported that they obtained the largest number of specimens in August among five sampling. They suggested the occurrence of aggregation of amphioxus for reproduction and they happened to hit such a patch during dredging. However, we never encountered such an aggregation to verify the presence of specific aggregation during the reproductive period.

Settling of young individuals

We observed large annual differences in the period of settling and in the number of settling individuals. In 1999, settling of young individuals was limited, while in 2000, a considerable number of young individuals settled. In 2001, settling occurred later than in the previous two years, and the number of young individuals was smaller than in 2000, but larger than in 1999. We assumed that the individuals in Group I were three years older than those of Group II. This assumption implies that settling was poor in the two years after Group I settled, and then in the third year a large number of young individuals settled that constituted Group II.

Xianhan *et al.* (1995) provided frequency histograms of *B. belcheri tsingtauense* at Quindao, which showed four age groups. Interestingly, a large group that consisted of fourth-year individuals dominated other age groups, suggesting that this group had settled extraordinarily well. Gosselck and Spittler (1979) studied the age structure and growth of *B. senegalense* in northwestern Africa. Despite negligible temperature fluctuations, reproduction was limited to a relatively short of only three months per year. They found that a sample collected in March 1972 contained no young individuals and a sample obtained on 22 June 1972 contained only five individuals of the youngest age group, among 785 animals. However, they showed that a three- to six-month-old group constituted a large portion of the samples obtained on 27 February 1976. These results suggested that settling was unsuccessful in 1972 but successful in 1975.

Settling of *B. floridae* has been studied in detail (Stokes, 1996; Stokes and Holland, 1996a, b). Spawning of this species continues from May to September at one- two-week intervals. Young individuals settle between May and October at intervals of one to three weeks. Samples obtained later in the breeding season contained numerous cohorts of settled individuals. In our study area, spawning likely occurs twice during the breeding season; however, almost all gametes are extruded by the first spawning between mid June and early July (see Part II). Therefore, the possibility of the appearance of two cohorts of settled individuals is negligible.

Sex ratio

We found that the sex ratio deviated from an expected 1:1 ratio among smaller individuals. This deviation was not large, but it was statistically significant, and it is interesting that the sex ratio was subsequently 1:1 for large individuals. In Fig. 8, the number of specimens differed between sexes; however, size frequency patterns were symmetrical, indicating that growth rates did not differ between the sexes. If there were differences in growth rates, we should see changes in the size-frequency distributions, with greater changes for large individuals, however, the patterns were almost identical. A larger mortality rate in males might have caused the rebalance in the sex ratio.

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