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Diurnal Spermatogenesis and Spawning in the Secondary Male of a Protogynous Wrasse, *Pseudolabrus japonicus* (Teleostei, Labridae)

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ABSTRACT—The bambooleaf wrasse, *Pseudolabrus japonicus*, exhibits diandric protogyny, and the secondary male performs pair spawning with a female who enters his territory. We examined diurnal spermatogenesis and spawning in the secondary male of a protogynous wrasse. In captivity, a single secondary male spawned daily over one month between 06:00 and 09:00 from October to November. Number of B-type spermatogonia and spermatocytes showed the lowest level at 00:00, increased gradually thereafter, peaked at 15:00, and decreased rapidly from 21:00 to 00:00. Spermatid number did not change significantly throughout the day. The number of spermatozoa increased gradually from 18:00, reached a maximum at 06:00, just prior to spawning, and thereafter decreased markedly at 09:00, after spawning. These results clearly showed that spermatogonial proliferation and meiosis occurred between 00:00 and 15:00, and spermiation occurred between 18:00 and 06:00. Thus, the secondary male of bambooleaf wrasse exhibits a diurnal rhythm of spermatogenesis and spermiation.

INTRODUCTION

Many marine teleost species undergo multiple cycles of gamete maturation and spawning within a single spawning season. Recently, some species have been reported as daily spawners, red seabream *Pagrus major* (Matsuyama *et al.*, 1988), Japanese whiting (kisu) *Sillago japonica* (Matsuyama *et al.*, 1990), tobinumeri dragonet *Repomicenus beniteguri* (Zhu *et al.*, 1989), and bambooleaf wrasse *Pseudolabrus japonicus* (Matsuyama *et al.*, 1997a, b). Daily spawning has some advantages for investigations into the mechanism of gamete formation; e.g., physiologically different germ cells can be obtained on the same day as demand dictates. However, studies on short-term cyclical changes in the gonad have been focused on the ovary, and testicular activity of male fish during the spawning season has not been investigated.

The bambooleaf wrasse is among the most numerous labrids on coastal rocky reefs in the southern part of Japan. It exhibits diandric protogyny, with populations consisting of small initial-phase (IP) males (primary males), IP females (primary females), and large terminal-phase (TP) males which may be derived either from females which have undergone a sex change to become male (secondary males), or from IP males (TP primary males; Nakazono, 1979). TP males, whether of primary or secondary derivation, differ from IP males and females in behavior and coloration. This life cycle is typical of diandric wrasse (Warner and Robertson, 1978; Warner, 1984). TP males exhibit gaudy brown coloration, while IP fish are reddish. In this species, the spawning behavior is mainly pair spawning performed by a TP male and a female, and group spawning has not been observed (Nakazono, 1979). IP males sometimes rush in and release milt during pair spawning by TP males. IP males do not defend territories. In contrast, TP males defend individual spawning territories around the perimeter of the rocky reef, and spawn exclusively with one female between 08:00 and 10:00. After pair spawning with one female, the pair is dissolved and the TP male begins to show spawning behavior again with another female. Thus, the TP males mate with some of the females which visit the spawning site in one day. Furthermore, it has been suggested that a single TP male performs daily spawning during the spawning period, based on long term, not daily, underwater observation (Nakazono, 1979). Recently, we have reported the daily rhythm of spawning and oocyte development (Matsuyama *et al.*, 1997a), and the diurnal rhythm of steroid production involved in the daily final oocyte maturation in the female bambooleaf wrasse (Matsuyama *et al.*, 1997b). These studies clearly

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showed that daily spawning behavior regularly occurred between 06:00 and 09:00 (Matsuyama *et al.*, 1997a, b), and the former study, in which spawning was performed by a single female and two secondary males in captivity, suggested the possibility of daily spermatozoa release by the secondary male of this species (Matsuyama *et al.*, 1997a).

In this paper, we ask whether spermatozoa are produced and released daily by the TP males, or whether they store spermatozoa for future release. In other words, whether TP males exhibit a diurnal rhythm of spermatogenesis and spermiation. To answer this question, we firstly confirmed daily spawning during the spawning season by a single secondary male bambooleaf wrasse reared in captivity with two females (experiment 1). Next, we studied the time course of germ cell development in the testes of secondary males captured in the field during one day in the spawning season (experiment 2).

MATERIALS AND METHODS

Animals

Experiment 1: The spawning season of bambooleaf wrasse is relatively short, about two weeks from mid to late October (Nakazono, 1979). Fish were collected with hook and line on Sept. 30, 1993 in Ago Bay, Mie Prefecture, and brought to Mie University in Tsu City. A captive population consisting of one TP male and two IP fish, each was established in two round fiberglass tanks of 200 l capacity (tanks A and B), each of which was supplied with filtered recirculating sea water (ca. 14 l/min) and a concrete block as a shelter. Fish were kept under the natural daylength conditions and at natural water temperature, and were fed with naked live shellfish once a day. In tank B, the water temperature was raised to 20°C on Oct. 19 and fixed thereafter until the end of experiment. Spawning was monitored every day by examining eggs which overflowed from the tanks into collecting nets; the eggs were counted and the fertilization rate was determined. After the experiment, all fish were sacrificed on January 23, 1994, and their gonads were examined histologically in order to determine their sexuality. Histological analysis of the gonad revealed that two TP males (95.4 and 97.4 g in body weight) were secondary males and four IP fish weighing 29.6–40.3 g (35.0 ± 2.2 g, mean ± SEM) were females.

Experiment 2: TP males were collected by hook and line in coastal water near the Fisheries Research Laboratory of Mie University in Ago Bay from mid Oct. to early Nov. in 1993. They were collected at 09:00, 12:00, 15:00 and 18:00, transferred to the laboratory within 30 min after sampling. Five fish in each sample were sacrificed for study. The remaining fish in each sample were stored in an outdoor tank (2.0 × 1.5 × 0.5 m) with concrete blocks supplied as shelter. The tank was lighted entirely by natural light, with the direct sunlight intercepted by the roof and three walls of a shed. The inflow of running sea water varied between 30 and 60 l/min. Fish stored in the tank spawned daily from the day after collection. Five TP males stored in the outdoor tank were sampled at 21:00, 24:00, 03:00 and 06:00. Gonadal histology showed that one fish of 40 TP males sacrificed was a primary male, and consequently a total of 39 TP males weighing 43.1–127.7 g (84.9 ± 3.9 g) were used for the quantitative study of germ cell composition.

Terminology

The term "spermatogenesis" refers to the entire formative process of spermatozoa from spermatogonia. The term "spermiogenesis" refers only to the differentiation of spermatozoa from spermatids, and this process proceeds within the cyst. The term "spermiation" describes the terminal phenomenon of the spermatogenic cycle. Spermiation in the lobular type of testis involves the release of spermatozoa into the lobular lumen and their arrival in the sperm duct (Billard, 1986; Nagahama, 1986). In many species, expulsion of the spermatozoa from the sperm duct upon abdominal massage reveals the occurrence of this process. In bambooleaf wrasse, outflow of a small amount of milt through the genital pore as a result of massage is always observed during the spawning season. It is difficult, thus, to confirm the occurrence of spermiation in fish such as bambooleaf wrasse which produce only a small amount of milt during a short cycle. In the present study, therefore, we employed the method based on measuring the amount of spermatozoa released into the lobular lumen from the cyst in histological sections in order to confirm the occurrence of spermiation, instead of abdominal massage.

Quantitative analysis of germ cells

Prior to quantitative analysis of the germ cells, tissue from nine sites in the left testicular lobe (Fig. 1A) of a secondary TP male (body weight = 93.4 g) sampled at 15:00 was examined to assess the

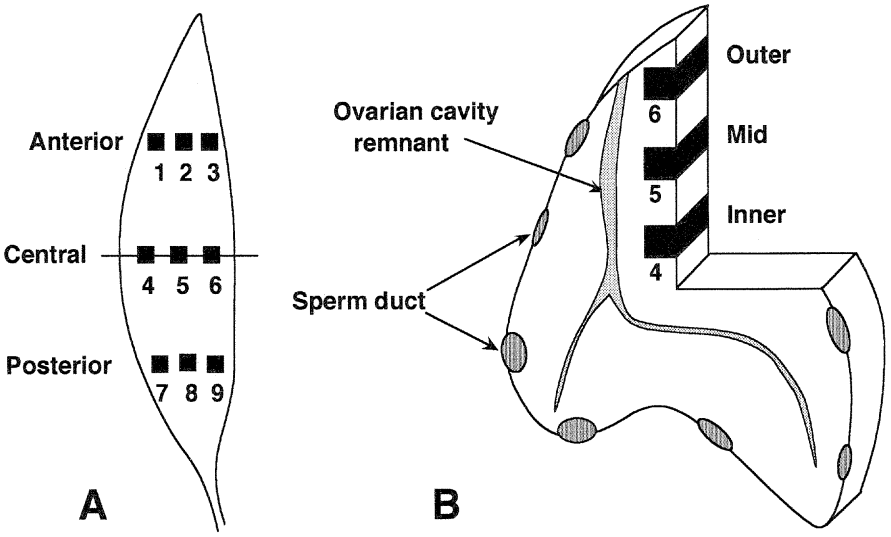

Fig. 1. (**A**) Left testicular lobe of secondary male bambooleaf wrasse, and nine subsamples of testicular tissue for quantitative analysis of germ cells, taken from the inner (sites 1, 4, and 7), mid (sites 2, 5, and 8), and peripheral regions (sites 3, 6, and 9). (**B**) Cross section of the central part of the left testicular lobe taken at the level indicated by the cross-line in **A**.

synchronicity of testicular germ cell development in different sites. There were significant differences in the number of germ cells (ANOVA, $p<0.05$) in the inner (sites 1, 4, and 7), mid (sites 2, 5, and 8) and peripheral regions (sites 3, 6, and 9), with a tendency toward an increase in number of spermatozoa in the mid region (Table 1). Therefore, tissue for the quantitative study of germ cells was sampled from site 5 (Fig. 1B) of the left lobe. The tissue samples were immersed in Bouin's solution, dehydrated, and embedded in Technovit resin (Kulzer & Co, GER). For light microscopy, 2 μm-thick sections were cut horizontally parallel to the longitudinal axis of the testis (Fig. 2A) and stained with a 1% solution of toluidine blue. Ten cross sections of different seminal lobules were selected randomly from each sample. The types of testicular germ cells were classified according to Miura *et al.* (1991). Type A and early type B spermatogonia were morphologically similar, with clear homogenous nuclei containing one or two nucleoli. As the proliferation of spermatogonia progressed, late type B spermatogonia which had a dense and heterogeneous nucleus appeared. Under the lightmicroscopical observation, it is difficult to distinguish morphologically between late type B spermatogonia and spermatocytes, although fine structures were different from each other; e.g., spermatocytes is characterized by a nucleus with synaptnemal complexes. In the present study, therefore, late type B spermatogonia and spermatocytes were combined as a cell type "B-type spermatogonia and spermatocytes". Then, three types of testicular germ cell (B-type spermatogonia and spermatocytes, spermatids, and spermatozoa) appearing in these cross sections (Fig. 2B) were counted and the number of each cell type per cross section of a seminal lobule were calculated. Data were analyzed statistically using Duncan's multiple range test.

Table 1. Number of spermatozoa per cross section of seminal lobule in the nine sites of the left testicular lobe

Inner site	Mid site	Peripheral site
1. 81.3±24.6	2. 113.3±34.3	3. 99.6±27.1
4. 72.8±22.1	5. 91.3±43.1	6. 54.6±18.7
7. 75.4±23.6	8. 106.8±26.2	9. 70.2±28.4

See Fig. 1 for the nine sites in the left testicular lobe. Values are mean ± SEM of the number of spermatozoa in ten cross sections of different seminal lobules.

RESULTS

Testicular structure of the secondary male

The paired testes are elongate white organs which lie in the body cavity and are attached to the ventral surface of the swim bladder by mesorchia. Testes of the secondary males lacked a central sperm duct. Newly formed efferent sperm ducts were situated longitudinally along the testicular wall where the open ends of the lobules converge in the testis. Efferent sperm ducts existed all along the testis but in no case were they used as a storage organ in the secondary males of bambooleaf wrasse. A remnant of the membrane bound ovarian cavity still remained (Fig. 3).

Testicular structure in teleosts varies from one species to another, although two basic types, tubular and lobular, can be identified according to the differentiation of the germinal tissue (Billard, 1986; Nagahama 1983, 1986). The testis of the secondary male of bambooleaf wrasse belongs to the latter type.

Experiment 1

A record of the number of eggs obtained from a single secondary male and two females is shown in Fig. 4. The first spawning occurred in both tanks on Oct. 1, 1993, the day after collecting the fish and the start of the experiment.

In tank A, which was kept under natural conditions, daily spawning continued up to mid November, and the last spawning was observed on Nov. 26. There was a tendency for the number of eggs spawned to decrease as the water temperature declined. The fertilization rates of eggs spawned varied from 0 to 100% (54.9 ± 4.1%).

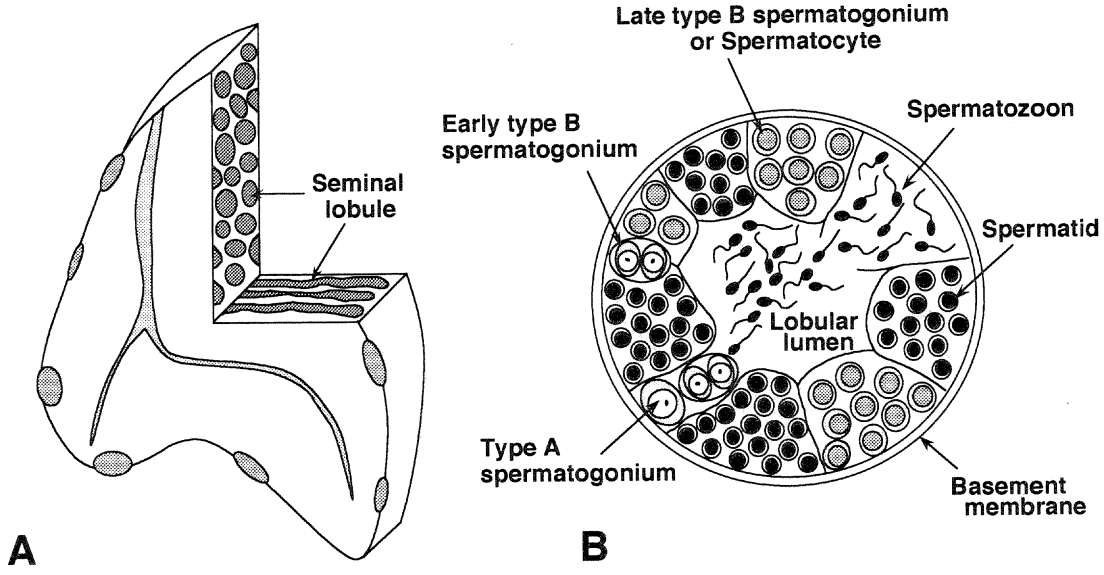

Fig. 2. (**A**) Sagittal and horizontal sections of left testicular lobe of a secondary male bambooleaf wrasse. The sagittal section shows cross sections of seminal lobules. (**B**) Cross section of a seminal lobule. See text for morphological characteristics of each germ cell stage.

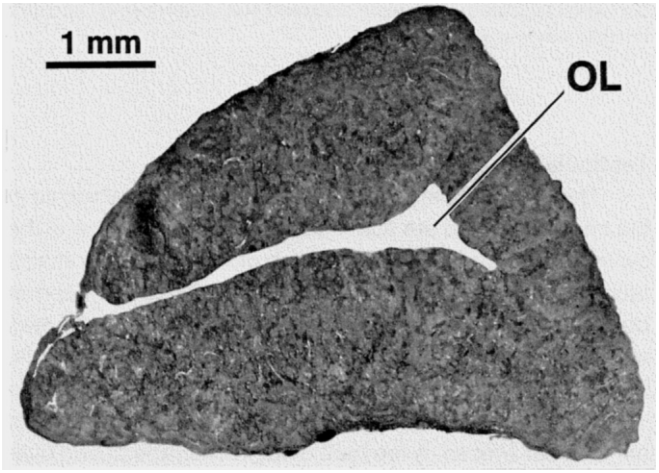

Fig. 3. Light micrograph of the testis of secondary male bambooleaf wrasse, showing the remnant of the membrane bound ovarian lumen (OL).

In tank B, two series of daily spawning were observed. The first one occurred from Oct. 1 to 18. The gradually decreasing water temperature was stabilized at 20°C on the next day (Oct. 19) and thereafter. The second series of spawning occurred on Nov. 14 and continued until Dec. 28. The fertilization rate of eggs spawned in the early series were higher ($81.0 \pm 6.4\%$) than that in latter one ($43.7 \pm 4.4\%$).

From these results, it was confirmed that a single secondary male of bambooleaf wrasse spawned daily during the spawning season.

Experiment 2

Changes in germ cell composition investigated at 3 hr intervals are shown in Fig. 5. The number of B-type spermatogonia and spermatocytes was at its lowest (199 cells/lobule) at 00:00 ($p<0.01$ vs. 15:00 and 21:00), increased gradually thereafter, peaked (294 cells/lobule) at 15:00 ($p<0.01$ vs. 0:00; $p<0.05$ vs. 6:00), and decreased rapidly from 21:00 (290 cells/lobule) to 00:00. Spermatid number exhibited a pattern similar to that of B-type spermatogonia and spermatocytes, i.e. reaching the lowest level at 03:00 (244 cells/lobule), peaking at 18:00 (308 cells/lobule), and decreasing from 21:00 (308 cells/lobule) to 00:00 (263 cells/lobule). However, there was no statistically significant change throughout the day. At 06:00, just prior to spawning, the lobular lumens were occupied with a large number of spermatozoa which had been released from the cysts (Fig. 6A), the highest level (262 cells/lobule) seen throughout the day ($p<0.01$ vs. 09:00 and 18:00; $p<0.05$ vs. 00:00, 12:00, 15:00 and 21:00). At 09:00, after spawning, spermatozoa showed a marked decrease in number (92 cells/lobule, $p<0.01$ vs. 06:00), when only a relatively small amount of spermatozoa was present in the lobular lumen (Fig. 6B). Thereafter spermatozoa remained at constant low levels for a long period, between 09:00 and 00:00. At 18:00, the spermatozoa number reached its lowest level (84 cells/lobule, $p<0.01$ vs. 06:00).

These results clearly show that spermatogonial proliferation (mitosis) and meiosis occurred between 00:00 and 15:00, followed by spermiation (release of spermatozoa into the lobular lumen from the cysts) between 18:00 and 06:00, immediately prior to spawning.

DISCUSSION

The histological characteristics of the testes of secondary males were similar to those of other diandric wrasses (reviewed by Sadovy and Shapiro, 1987). Primary and secondary males exhibited distinct differences in gonad size and gross morphology; primary males were generally smaller than secondary males, but they had much larger gonads and mean gonadosomaic index (GSI) (data not shown). The testes of primary males had a single centrally located tubular sperm duct and no remnant of ovarian lumen was observed. On the contrary, in the secondary males newly formed efferent sperm ducts were arranged longitudinally along the testicular wall. However, the pattern of germinal tissue differentiation in both types of male was similar. Both belonged to the lobular type (Billard *et al.*, 1982; Nagahama, 1983, 1986), in which numerous separate seminal lobules radiate from the central sperm duct (in the case of primary males' testes) or to the peripheral sperm ducts (in secondary males' testes). As spermatogenesis proceeds, the cysts expand and eventually rupture, liberating spermatozoa into the lobular lumen. Although peripherally situated sperm ducts in the testes of secondary males were filled with spermatozoa, there were no sperm storage organ in the testes, as has been observed in other species such as Japanese flounder *Paralichthys olivaceus* (Matsuyama *et al.*, 1995). Therefore, recruitment of spermatozoa from the cysts *via* the lobular lumen may directly reflect the volume of milt released from genital pore.

The results from Experiment 1 clearly showed that a single secondary male releases milt daily in the spawning season. In tank A the number of eggs spawned tended to decrease as the water temperature declined, and spawning stopped when the temperature reached around 15°C. Our previous study (Matsuyama *et al.*, 1997a), in which a single female was reared with two secondary males, also gave a similar result, suggesting that 15°C is the lower limit of temperature for spawning of bambooleaf wrasse. In tank B the temperature was fixed at 20°C at the end of a series of daily spawning, when the temperature had declined to 17°C, and the second series of daily spawning started about four weeks later and continued for 44 days. These results suggest that temperature may play a role in terminating the spawning season of the bambooleaf wrasse.

To our knowledge, the first record of continuous daily spawning by a single male fish is Egami's study of medaka *Oryzias latipes* (Egami, 1959). Thereafter, in the last two decades, marine biologists have studied the mating behavior of a wide variety of reef species in their habitats. Consequently, daily spawning by a single male fish seems sometimes to be common among the protogynous species in which larger males

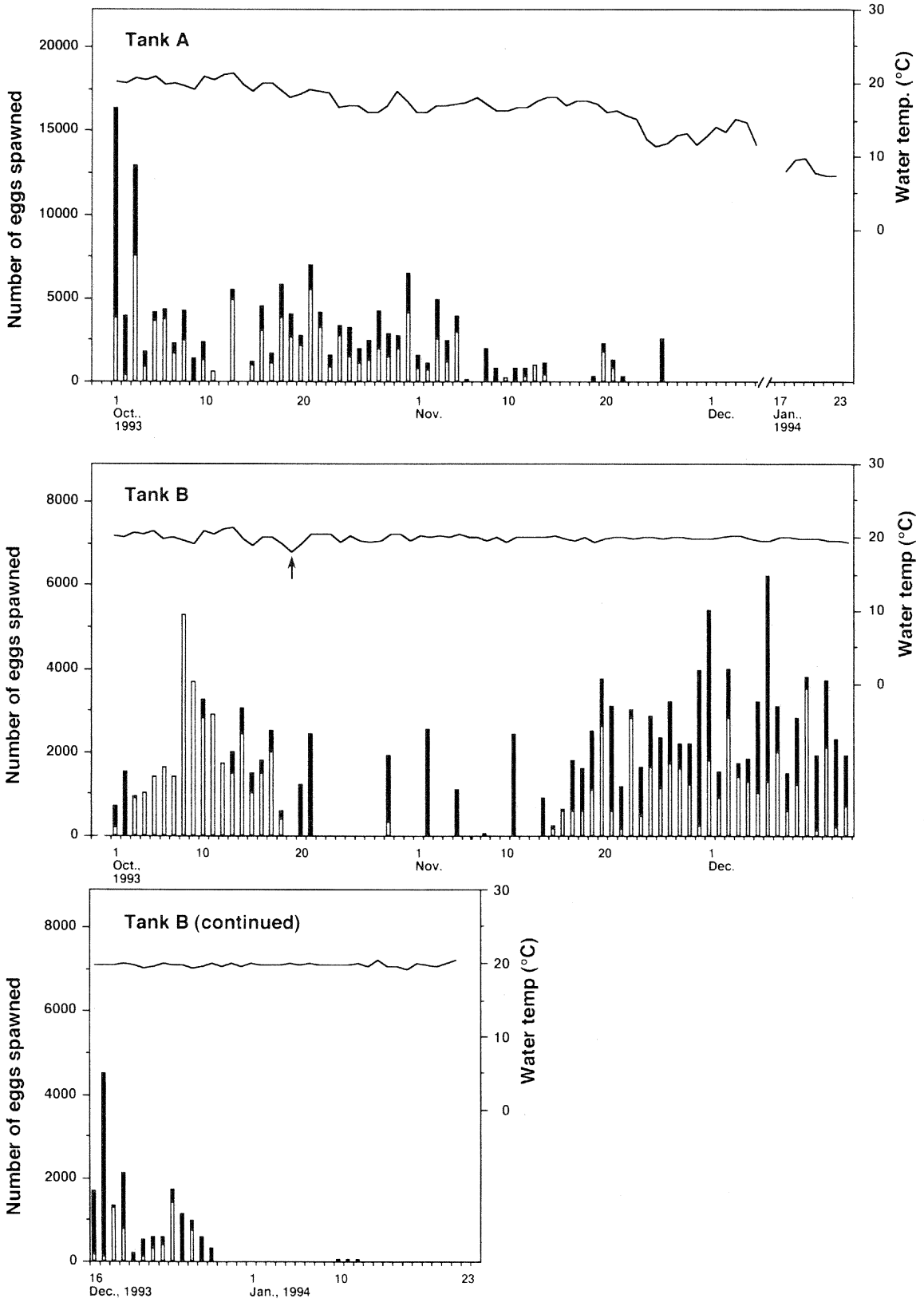

Fig. 4. Number of eggs spawned by a single secondary male and two females of bambooleaf wrasse. Tank A was kept under natural photoperiod and temperature conditions. In tank B, the water temperature was fixed at 20°C from October 19, 1993. Open and solid bars show floating (almost fertilized) and sinking (unfertilized) eggs, respectively.

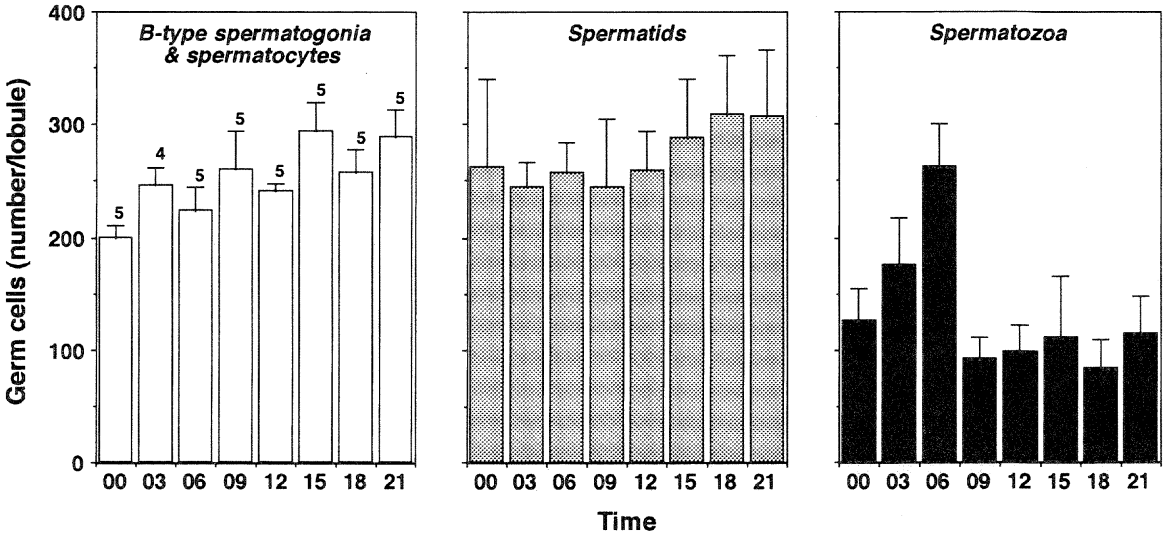

Fig. 5. Diurnal changes in the number of different testicular germ cells per cross section of seminal lobule. Numerals above the bars show sample size, and values are mean ± SEM of each sample.

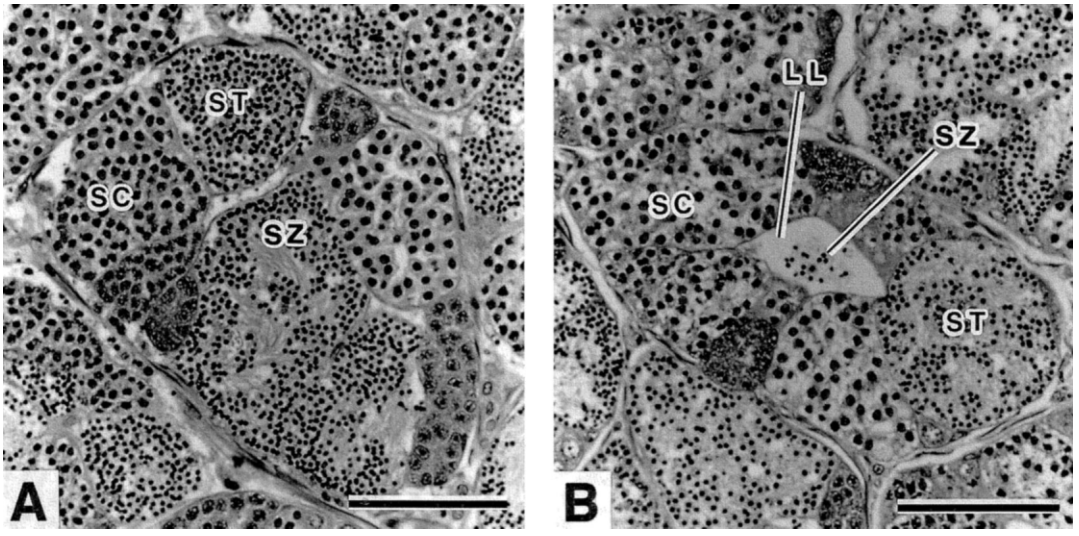

Fig. 6. Cross section of the seminal lobules of testes of secondary male bambooleaf wrasse sampled at 06:00 (**A**) and 09:00 (**B**). Note the large amount of spermatozoa in the lobular lumen released from cysts at 06:00 (**A**) just prior to spawning, and the decreased number of spermatozoa in the lobular lumen at 09:00, due to spawning (**B**). SC and ST represent spermatocytes and spermatids within each cyst, respectively. SZ, spermatozoa released into the lobular lumen. LL, lobular lumen. Scale bars represent 50 μm.

tend to monopolize mating, either by defending spawning sites that the females visit or by controlling a harem of females (Warner, 1984). Daily spawning is found in a diverse array of families such as wrasses, angelfishes, bass, and sandperches (see, for example, Robertson, 1972; Moyer and Nakazono, 1978; Yogo, 1985; Nakazono *et al.*, 1985). As described above, it has been shown that a single male fish of some species spawns daily during the spawning season; however, there has been no investigation of the diurnal rhythm of gamete formation in male fish.

The results from Experiment 2 demonstrated that spermatogenesis of secondary male bambooleaf wrasse occurs on a daily basis; in other words, the secondary male of bambooleaf wrasse has a diurnal rhythm of spermatogenesis. We previously referred to the diurnal rhythm of spermatogenesis in Japanese flounder (Matsuyama *et al.*, 1995), in which the method for analysis of change of germ cell number in the testis was the same as in the present study. That study, however, lacked an experiment to prove daily milt release by a single male in captivity, which was included in the present study. Therefore, the present study is the first report which clearly shows the existence and dynamics of the diurnal rhythm of spermatogenesis in teleosts. Diurnal changes in germ cell composition in the seminal lobule are summarized in Fig. 7.

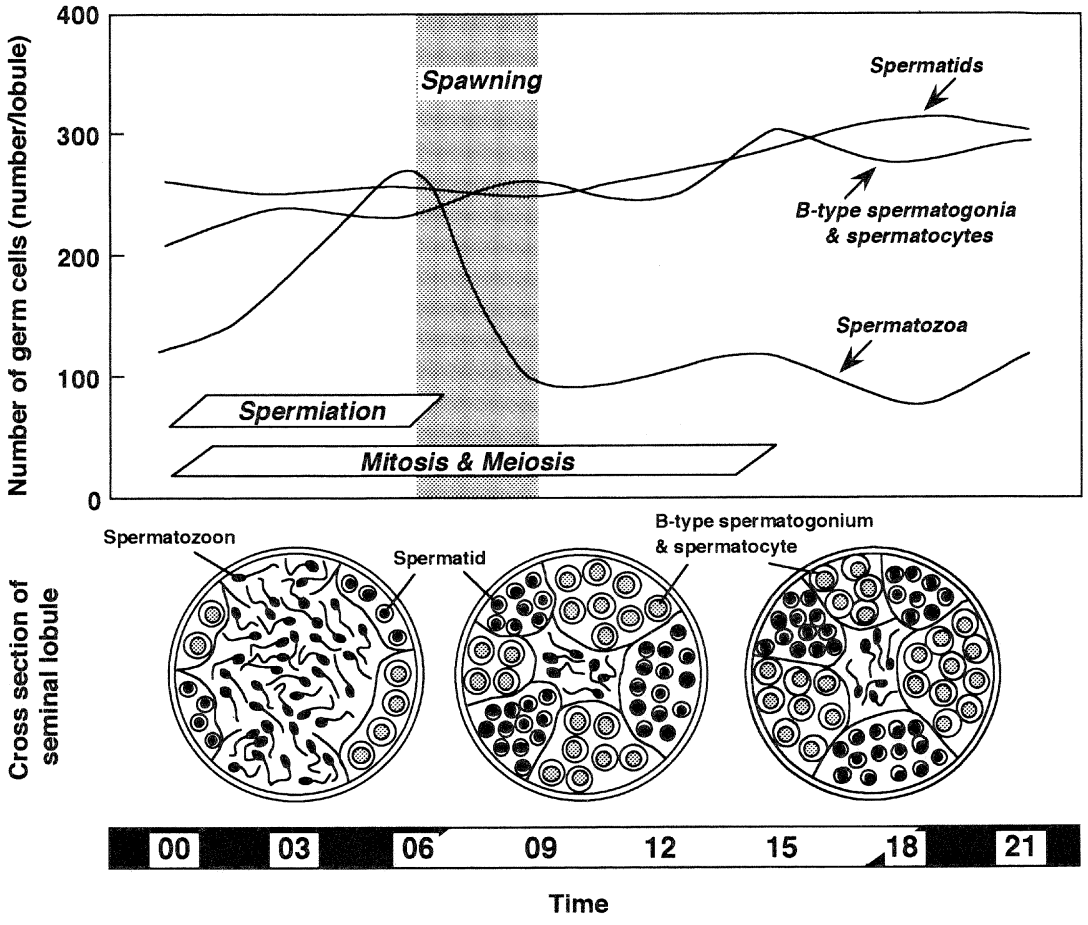

Fig. 7. Diagrammatic representation of the diurnal changes in testicular germ cell number in the secondary male bambooleaf wrasse. B-type spermatogonium in the cross section of a seminal lobule means late type B spermatogonium reported by Miura *et al.* (1990); see text for morphological characteristics of each germ cell stage. Black and white parts in the column showing time represent night and day, respectively.

The present study clearly showed that spermiation occurs between 18:00 and 06:00, and the large number of spermatozoa in the lobular lumen drastically decreased at 09:00, due to spawning. These results agreed with the previous studies on spawning time of the female bambooleaf wrasse (Matsuyama *et al.*, 1997a, b), in which daily ovulation and spawning were observed between 06:00 and 09:00. Furthermore, spermatogonial proliferation (mitosis) and formation of spermatocytes (meiosis) occurred between 00:00 and 15:00. Thus, it is likely that daily-spawned spermatozoa were supplemented from spermatids between 18:00 and 06:00, and newly formed spermatocytes also developed to spermatids daily. In the present study, however, the duration of the life of each kind of testicular cell was unclear. The spermatogenic events in medaka have been studied autoradiographically following administration of tritiated thymidine, and at 25°C the length of life of spermatocytes (between pre-leptotene and spermatid) and spermatids (between first meiotic metaphase and spermatozoa) was 5 and 9 days, respectively (Egami and Hyodo-Taguchi, 1967). The testicular structure of medaka is the tubular type, in which spermatozoa are grouped into bundles called spermatozeugmas (Billard, 1986). The relatively long life of medaka spermatids may be due to the formation of spermatozeugmas. Recently, the entire process of spermatogenesis has been induced to occur *in vitro* by hormonal treatment in the Japanese eel *Anguilla japonica* (Miura *et al.*, 1991), the testis of which belongs to the lobular type. In induced spermatogenesis in the presence of 10 ng/ml 11-ketotestosterone *in vitro*, zygotene spermatocytes of the meiotic prophase occurred in testes cultured for 18 days, and after 21 days spermatids and spermatozoa were observed for the first time. These results suggest that spermiogenesis of Japanese eel *in vitro* is approximately 3 days. In male domestic fowls, which are well known to be daily spawners, incorporation of tritiated thymidine in the testis has been measured autoradiographically at 3-hr intervals (Schanbacher *et al.*, 1974). Uptake of tritiated thymidine did not differ between day and night, suggesting that spermatogenic DNA production may not vary diurnally. Further studies using such autoradiographic or *in vitro* culture methods are necessary to investigate the minute kinetics of diurnal spermatogenesis, including the duration of the life of each testicular germ cell in bambooleaf wrasse.

In conclusion, the present study demonstrated that single secondary males of bambooleaf wrasse spawn daily during

the spawning season. It also showed the existence of a clear diurnal rhythm of spermatogenesis and spermiation. Although it is generally accepted that the principal stimuli for vertebrate spermatogenesis are pituitary gonadotropins and androgens, the specific role played by individual hormones has not been clarified (Steinberger, 1971; Callard *et al.*, 1978; Billard *et al.*, 1982; Hansson *et al.*, 1976). In teleosts, testosterone and 11-ketotestosterone have been implicated in the regulatory process of testicular development, and 17α,20β-dihydroxy-4-pregnen-3-one, the oocyte maturation-inducing hormone (MIH) in many teleost species (Scott and Canario, 1987), is thought to be responsible for spermiation in male teleosts (Nagahama, 1987; Fostier *et al.*, 1987). Hormonal profiles and *in vitro* steroid production of testicular tissue during daily spawning in the secondary male of bambooleaf wrasse are now being examined by us. The diurnal rhythms of spermatogenesis and spermiation make this species a good model to investigate the mechanism of spermatozoa formation, and should provide interesting informations in the future.

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