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Aggregating Behavior of the Grass Puffer, *Takifugu niphobles*, Observed in Aquarium during the Spawning Period

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The grass puffer (*Takifugu niphobles*) exhibits unique spawning behavior. Large numbers of fish aggregate to coastal spawning beds several hours before high tide during the spring tide. In order to examine the environmental and physiological regulation of this semilunar-synchronized spawning rhythm, the aggregating and spawning behaviors of the grass puffer were observed in the field, and in an aquarium without tidal changes. The fish aggregated to a spawning bed in the rising tidal phases both in the morning and evening during the spring tide, and several days after the spring tide. Spawning occurred on several days when large numbers of fish (200–1000) aggregated to the spawning bed. The timing of aggregation and spawning was tightly connected to the tidal changes; aggregation occurred 2–3 h before high tide, and spawning occurred 2 h before high tide. In the aquarium, in which a slope was constructed with pebbles, small groups of mature fish aggregated on the slope only in the rising tidal phases during and after the spring tide, when the fish aggregated in the field. However, there was no spawning in the aquarium. The aggregating behavior observed in the aquarium without tidal changes suggests that the semilunar reproductive rhythm is endogenously maintained with surprising precision during the spawning period in grass puffer.

Key words: Puffer, Reproductive behavior, Semilunar cycle, Spawning rhythm, Zeitgeber

INTRODUCTION

The synchronization of reproductive functions is crucial to reproductive success in most vertebrate species. In fishes living in temperate to polar zones, day-night cycles are accompanied by periodic changes in the light and water temperature of aquatic environments. These environmental factors are often utilized by the fish as a forcing oscillation that entrains a biological rhythm (often called a *Zeitgeber*) for the synchronization of various reproductive activities. Changes in moonlight and tide are used as the major *Zeitgeber* by various species of fish living in shallow waters and reef areas (Leatherland et al., 1992; Takemura et al., 2004b). These fishes exhibit lunar- or semilunar-related changes in their physiological and behavioral activities.

Lunar and semilunar reproductive cycles have been reported in many marine teleost species, such as the California grunion (*Leuresthes tenuis*) (Clark, 1925), damselfish (*Pomacentrus nagasakiensis*) (Moyer, 1975), mummichog (*Fundulus heteroclitus*) (Tayler et al., 1979; Hsiao and Meier, 1989), groupers (Collin et al., 1987; Sadovy et al., 1994), rabbitfishes (Hoque et al., 1998; Rahman et al., 2000; Takemura et al., 2004) and mudskipper (*Bolephthalamus perctinirostris*) (Wang et al., 2008). Such reproductive cycles are solely dependent on the position of the moon relative to the earth and sun, and changes in moonlight and/or tides are possible environmental cues for the synchronization (Leatherland et al., 1992). Several studies under laboratory conditions suggested that lunar-related rhythms are endogenously maintained (Hsiao and Meier, 1986, 1989; Hoque et al., 1998; Rahman et al., 2000). In order to determine whether reproductive cycles are synchronized by moonlight or tidal cues, Hsiao and Meier (1989) examined the semilunar reproductive cycles of two closely related killifish, *F. grandis* and *F. heteroclitus*, which subsist in Gulf habitats with different tidal cycles. It was suggested that the environmental synchronizers were different in these two species: That of *F. grandis* was related to the tidal cycle, while that of *F. heteroclitus* was related to both the moonlight and tidal cycles. In rabbitfishes, the moonlight cycle was crucial for their lunar-synchronized spawning cycles (Takemura et al., 2004a, 2004b, 2006). Therefore, the environmental synchronizers seem to be different depending on species and environmental conditions.

The grass puffer (*Takifugu niphobles*) is a common intertidal puffer species in Japan, and it spawns in semilunar cycles during the spring tide from spring to summer. These fish aggregate at certain seashore sites several hours before high tide at dusk, and spawning usually continues for 1–2 hours during the rising tidal phase (Uno, 1955; Katayama and Fujita, 1967; Nozaki et al., 1976; Kobayashi

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et al., 1978; Tsutsumi, 1978; Suzuka and Isogai, 1979; Honma and Kitami, 1980; Honma et al., 1980; Yamahira, 1994, 2004). Spawning takes place in groups of 10–60 individuals, of which one is female. Before spawning, several tens of males actively pursue one female. Since the spawning occurs at the same location every year, and every two weeks during a spawning season (Nozaki et al., 1976), we are aware of time and place of the spawning and thus can make careful and extended observations of spawning behavior and obtain spawning fish for behavioral and physiological studies. Grass puffer, therefore, provides a useful model for examining the environmental and physiological regulation of the lunar-synchronized spawning rhythm.

Since the spawning occurs during the rising tide at dusk, it seems that both tidal and diurnal cycles are involved in the spawning rhythm. However, the environmental cues for the synchronization of spawning behavior are largely unknown. Honma et al. (1980) showed that there was little rhythmicity in the spawning of grass puffer on the coast of Sado Island, where tidal fluctuation is very low, only 0.2 to 0.3 m in each cycle, suggesting that tidal changes are more important for the spawning rhythm than changes in moonlight. The present study was designed to clarify the environmental cues important for the entrainment of the semilunar-synchronized spawning rhythm in grass puffer. We examined the spawning rhythm of grass puffer in the field, and also transferred fish to an aquarium during the spawning period and made careful observations of their behavior under laboratory conditions without tidal stimuli.

MATERIAL AND METHODS

Field observations of spawning behavior

Field observations of spawning behavior of grass puffer were carried out during the spawning period from the end of April to the beginning of June from 2007 to 2009 in Shikanoshima Island, Fukuoka, Japan (Kurose beach, 33°69' N, 130°30' E). The tidal fluctuation is about 2.2 m in each cycle. The observations were made in the dawn-morning and dusk-evening rising tidal phases for usually 5–8 days around the spring tide. The approximate number of the fish that appeared at the spawning bed, the time of the start, and duration of spawning were recorded.

Animals used for observations in aquarium

Mature grass puffer of both sexes (42.5–94.1 g in body weight, 9.9–15.4 cm in folk length) were collected at two spawning grounds, Murotsu, Yamaguchi, Japan (34°14' N, 130°89' E) and Shikanoshima Island (Kurose beach) during the spawning period. In Murotsu, the fish were caught by settled net on April 30, 2009. In Shikanoshima Island, the fish that appeared on Kurose beach were caught by dip net on May 11 and May 27, 2009. The fish were transferred to the Fishery Research Laboratory station, Kyushu University, Fukutsu, Japan (33°47' N, 130°27' E), and were kept in indoor tanks (500 l) with the flow of seawater at 17–20°C under nat-

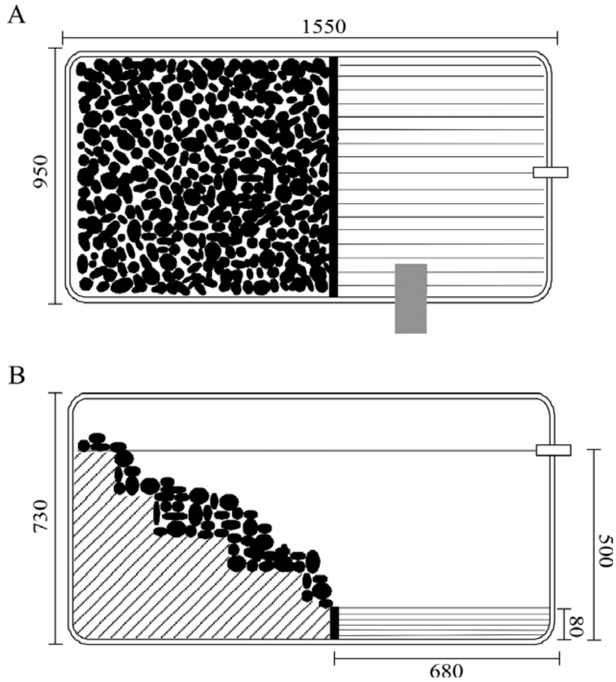

Fig. 1. Schematic illustrations of the experimental aquarium. **A**, horizontal view; **B**, lateral view. A slope was constructed with pebbles (black circles) and plastic containers (diagonal lines). Sand is shown as horizontal lines. A gray box indicates a *shishi-odoshi*, which is a length of pipe that slowly fills with seawater and then empties out. An open box indicates a drainpipe. The aquarium was filled with seawater 50 cm in depth. The length is shown in mm.

Table 1. The date and time of recording of behavior.

Recording No.	Date	Tide	Age of the moon	High tide (Morning)	Low tide	High tide (Evening)	Time of recording	Rising or ebb tide[1]	Aggregation behavior in field[2]	Origin of fish used in the recording
1	10 May	Spring tide	15.0	**9:47**[3]	16:24	22:56	8:10–9:40	Rising	+	Murotsu, 30 Apr.
2	24 May	Spring tide	29.0	**9:01**	15:36	22:08	7:30–9:00	Rising	+	Kurose, 11 May
3	25 May	After spring tide	0.6	**9:43**	16:24	22:54	8:10–9:40	Rising	+	Kurose, 11 May
4	26 May	After spring tide	1.6	**10:26**	17:15	23:40	9:00–10:30	Rising	+	Kurose, 11 May
5	26 May	After spring tide	1.6	10:26	**17:15**	23:40	15:45–17:15	Ebb	–	Kurose, 11 May
6	1 Jun.	Neap tide	7.6	4:11	**10:38**	16:47	9:00–10:30	Ebb	–	Kurose, 27 May
7	1 Jun.	Neap tide	7.6	4:11	10:38	**16:47**	15:00–16:30	Rising	–	Kurose, 27 May
8	2 Jun.	Neap tide	8.6	5:16	**11:52**	18:10	10:20–11:50	Ebb	–	Kurose, 27 May
9	2 Jun.	Neap tide	8.6	5:16	11:52	**18:10**	16:50–18:20	Rising	–	Kurose, 27 May
10	8 Jun.	Spring tide	14.6	**9:26**	16:15	22:44	8:00–9:30	Rising	–	Kurose, 27 May
11	9 Jun.	Spring tide	15.6	**10:02**	16:49	23:19	8:30–10:00	Rising	–	Kurose, 27 May
12	10 Jun.	After spring tide	16.6	**10:39**	17:23	23:53	9:09–10:39	Rising	–	Kurose, 27 May

[1] Tide conditions during the time of recording.
[2] +, Aggregation behavior was observed in the field during the time of recording; –, Aggregation behavior was not observed.
[3] Time in bold indicates that the recording was made for 90 min before this time.

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ural photoperiod for 1–3 days.

Aquarium and rearing conditions

For observations of spawning behavior, 10 males and 2 females were selected by gently pushing the abdomen, and were placed together in an experimental aquarium (1 t, Fig. 1). The aquarium was filled with sand 8 cm in thickness across approximately half of its bottom area. In the other half of the tank, a slope was constructed by accumulating plastic containers and pebbles 5–10 cm in diameter to mimic the slope of the spawning beach. The aquarium was filled with seawater 50 cm in depth (approximately 700 l) with an on-and-off flow of 700 ml per 20 s produced by a *shishi-odoshi*, which is a length of pipe that slowly fills with seawater and then empties out. The temperature of seawater was 18–21°C. The aquarium was placed by the window to maintain a natural photoperiod (LD 14:10). Light intensity ranged from 0.1 lx (at midnight) to 7050 lx (in the daytime) at water surface. The fish were fed commercial pellets equivalent to 0.2–0.3% of body weight daily.

Video-recording and analysis of behavior

After acclimation to the experimental aquarium for 4–9 days, the behavior of twelve fish was recorded by a digital video camera (DCR-TRV7, Sony) placed above the aquarium. The recording time was 90 min during the rising tidal phases in the morning and evening of the spring and neap tides, and the ebb tidal phases in the spring and neap tides (Table 1).

On the basis of our preliminary observations in 2008, in which the behavior of mature fish were observed visually using the same aquarium, we classified the behavior of grass puffer in four categories: 1) halt on the slope of pebbles, 2) halt on the sand, 3) swim about and, 4) hide in the sand. In total, 18 h of recorded video was replayed (Table 1) and the number of fish in each category was counted every 5 min. The sum of these numbers in each observation (90 min) was used for construction of a bar chart.

Statistical analysis

The relative proportion of the fish that halted on the pebbles (category 1) were analyzed by a Student's t-test to detect statistically significant differences between the recordings of the rising tidal phases during and after the spring tide, when the fish aggregated in the field, and those of other time points.

RESULTS

Spawning behavior at Shikanoshima Island

Field observations of spawning behavior were carried out at Kurose beach in Shikanoshima Island during the spawning period from the end of April to the beginning of June. The beach, which consisted mainly of pebbles and boulders, is only 20 m wide along the coast. A number of mature fishes came to the beach for spawning only among the tiny and moderate waves. During the rising tide 2–3 h before the high tide, the fish aggregated to the beach and swam back and forth along the beach. Before spawning, they gathered and lined the edge of the water and floated on the sea, facing landward, and the males rarely chased the females. They rapidly stranded themselves on the beach with the wave action and flopped back into the wash of the next wave. On repeating this behavior several times, they suddenly released eggs and sperm, and the water was found stained with milt ejaculated by the males. The spawning occurred spontaneously along the beach for about 1 h.

Table 2. Aggregating and spawning behaviors in Shikanoshima Island in 2007–2009.

Rising tide	Year	Date	Age of the moon	Sun rise/ Sun set	High tide (a)	No. of fish[1]	Start of spawning (b)	Time between a and b
Morning	2007	30 Apr.[2]	12.6	5:30	8:04	100	–[3]	–
		1 May	13.6	5:29	8:29	50	–	–
		2 May	14.6	5:28	8:51	100	–	–
		3 May	15.6	5:27	9:11	200	8:20	0 h 51'
		4 May	16.6	5:26	9:31	>1000	7:40	1 h 51'
		5 May	17.6	5:26	9:54	>1000	7:40	2 h 14'
		6 May	18.6	5:25	11:15	>1000	10:15	1 h 00'
		7 May	19.6	5:25	11:51	100	–	–
		15 May	27.6	5:19	8:21	300	–	–
		16 May	28.6	5:18	8:59	400	–	–
		17 May	0.3	5:17	9:37	50	–	–
		18 May	1.3	5:17	10:16	500	–	–
		20 May	3.3	5:15	11:35	200	–	–
		2 Jun.	16.3	5:10	9:55	300	–	–
		3 Jun.	17.3	5:09	10:20	500	–	–
	2008	4 May	28.0	5:27	7:56	300	–	–
		5 May	29.0	5:25	8:31	500	7:05	1 h 26'
		6 May	0.6	5:26	9:08	>1000	–	–
		7 May	1.6	5:25	9:46	>1000	8:00	1 h 46'
		8 May	2.6	5:22	10:26	>1000	8:30	1 h 56'
		19 May	13.6	5:16	8:14	100	–	–
		20 May	14.6	5:15	8:45	600	–	–
		21 May	15.6	5:14	9:17	100	–	–
		6 Jun.	2.3	5:07	9:38	50	–	–
	2009	9 May	14.0	5:23	9:16	500	–	–
		10 May	15.0	5:22	9:47	>1000	7:40	2 h 07'
		11 May	16.0	5:21	10:19	600	8:00	2 h 19'
		12 May	17.0	5:21	10:51	600	8:00	2 h 51'
		13 May	18.0	5:20	11:24	200	–	–
		14 May	19.0	5:19	12:00	500	–	–
		22 May	27.0	5:14	7:40	100	–	–
		23 May	28.0	5:13	8:20	20	–	–
		24 May	29.0	5:13	9:01	30	–	–
		26 May	1.6	5:12	10:26	>1000	8:50	1 h 36'
		27 May	2.6	5:12	11:12	700	8:30	2 h 42'
Evening	2007	30 Apr.	12.6	18:59	20:37	30	–	–
		1 May	13.6	19:00	21:11	30	–	–
		3 May	15.6	19:02	22:16	300	–	–
		4 May	16.6	19:02	22:48	>1000	21:10	1 h 38'
		14 May	26.6	19:11	20:13	50	–	–
	2008	4 May	28.0	19:04	20:24	100	–	–
		6 May	0.6	19:05	22:00	100–300	–	–
		7 May	1.6	19:06	22:40	>1000	21:05	1 h 35'
		8 May	2.6	19:08	23:35	500	–	–
	2009	8 May	13.0	19:07	21:43	30	–	–
		9 May	14.0	19:07	22:20	500	–	–
		10 May	15.0	19:08	22:56	>1000	20:20	2 h 36'
		26 May	1.6	19:20	23:40	1000	–	–

[1] The approximate number of fish that appeared at the spawning site.
[2] The date with shadow indicates the spring tide.
[3] Hyphens indicate that no spawning occurred on the day.

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Fig. 2. Aggregating behavior of grass puffer in the aquarium. A slope was made of pebbles in the left half side. The right half side was filled with sand.

Timing of aggregating and spawning behaviors at Shikanoshima Island

The days when we could observe the aggregating fish at Kurose beach are listed in Table 2. On the other days around the spring tide, the fish did not come to the spawning bed. The fish aggregated to the spawning site with the semilunar cycle. They appeared on the beach in the rising tidal phases both in the morning and evening during the spring tide and several days after the spring tide. The number of observations of the aggregating fish was higher in the morning than the evening.

Spawning occurred over several days when large numbers of fish (200–1000) aggregated to the spawning bed. Spawning was commenced 2 h before the high tide (Average ± SEM = 1 h 54 min ± 9 min, n = 15, Table 2). In the evening, spawning occurred in the darkness after sunset.

Aggregating behavior in aquarium

In 2009, ten males and two females were left in the experiment aquarium, and their behaviors were observed by video-recording in the rising and ebb tidal phases during the spring and neap tides. They did not spawn in the aquarium at any time during the observation period. However, in the rising tidal phases during and after the spring tide, they aggregated on the slope of pebbles and halted there for about 1 h (Figs. 2 and 3A). They seemed to look at each other as they did at the edge of the water on the spawning beach before spawning. The proportion of this behavior decreased in the ebb tidal phases after the high tide (Fig. 3A, Recording No. 5) and in the neap tide (Fig. 3B). During the first spring tide in June, when the fish did not come to the spawning beach in the field (Table 2), no such aggregating behavior was observed in the rising tide (Fig. 3C). There was a significant difference in the proportion of the aggregating behavior between the recordings in the rising tidal phases during and after the spring tide (60.9 ± 10.2%, Recording No. 1–4) and those on other occasions (7.5 ± 4.4%, Recording No. 5–12) (Student's t-test, $p < 0.001$).

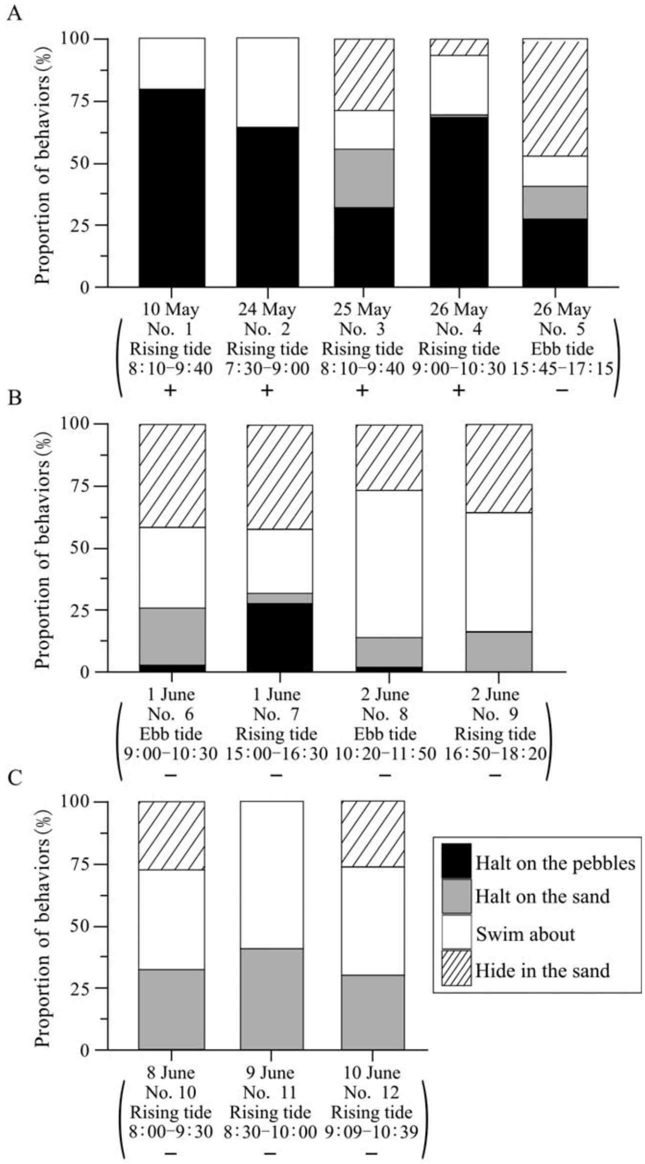

Fig. 3. Relative proportion of the four kinds of behaviors in the aquarium. The behavior was monitored during the spring tide (**A**), the neap tide (**B**) and the spring tide after the spawning period (**C**). For each observation, the recording number shown in Table 1, tide conditions, time of recording, and presence (+) or absence (–) of aggregation behavior in the field are indicated in parentheses.

DISCUSSION

In the present study, the aggregating and spawning behavior of grass puffers was observed in the field and the aquarium. Grass puffers aggregated to the spawning beach in the rising tidal phases both in the morning and evening during and after the spring tide. Spawning occurred on several days when large numbers of fish aggregated to the spawning bed. The timing of spawning was restricted to 2 h before high tide. In the experimental aquarium, small groups of mature fish aggregated on the slope of pebbles in the rising tidal phases during and after the spring tide, although the fish did not spawn in the aquarium. Furthermore, the aggregating behavior in the aquarium was observed only during the spawning period. These results suggest that grass puffer has endogenous semilunar and tidal cycles

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specific to the spawning period.

In the aquarium, ten males and two females halted on the slope for about 1 h during the rising tide. The males did not pursue females and stayed facing each other. This behavior resembles aggregating behavior in the field. Fish gathering on the beach lined and floated along the edge of the water before spawning, and spawning occurred spontaneously with wave action. This behavioral pattern is somewhat different from that observed in other groups of grass puffer, the males of which actively pursue a female (Uno, 1955; Katayama and Fujita, 1967; Nozaki et al., 1976; Kobayashi et al., 1978; Tsutsumi, 1978; Suzuka and Isogai, 1979; Honma and Kitami, 1980; Honma et al., 1980; Yamahira, 1994, 2004). As for the spawning of these groups of grass puffer, several stimuli of spawning behavior have been suggested, including 1) wave action (Uno, 1955; Nozaki et al., 1976; Suzuka and Isogai, 1979), 2) biting each other (Uno, 1955; Suzuka and Isogai, 1979), 3) joining other spawning groups (Suzuka and Isogai, 1979; Honma and Kitami, 1980) and, 4) sudden egg release by one female (Suzuka and Isogai, 1979). In the Shikanoshima group, however, physical stimulation between the males and the females is most unlikely, while stimulation by wave action may be a major trigger for spawning. It is therefore probable that the absence of spawning in the aquarium is due to the lack of the physical stimulation of the wave action. In the experimental aquarium, a *shishi-odoshi* was used to mimic wave action. However, this instrument might not be effective in triggering spawning behavior.

It is also possible that other conditions for rearing the fish are critical for inducing the spawning behavior, such as cyclic changes in hydrostatic pressure, the numbers of males and females in the aquarium, and structural characteristics of the slope and sand (Yamahira, 1997). The experimental fish were reared in the aquarium for 4–14 days before observation. We cannot neglect the possibility that this acclimation may affect spawning behavior but not aggregating behavior. Further studies will be needed to determine if mature grass puffers spawn in the rearing condition.

The aggregating behavior was observed in the aquarium under laboratory conditions without tidal changes. This behavior was observed only in the rising tidal phase during and after the spring tide when the fish aggregated to the spawning bed in the field. Field observation over three years revealed that the timing of aggregation and spawning behaviors was tightly connected to the tidal changes. Aggregation occurred 2–3 h before high tide and spawning occurred 2 h before high tide, suggesting that the commencement of these behaviors is determined by the high tide time but not the time of day (Yamahira, 1994, 2004). The vague periodicity of spawning of the fish at Sado Island, where the tidal fluctuation is very low (Honma et al., 1980), further supports the high contribution of the tidal cue to determination of the commencement of these behaviors. The present results, however, showed that these behaviors can occur in the aquarium without the tidal changes. These results suggest that the semilunar reproductive rhythm is endogenously maintained in grass puffer, and that the tidal cue is a crucial factor for entrainment of the rhythm in the field. It has been shown that the endogenous semilunar cycles are certainly synchronized by tidal and/or moonlight cues (Hsiao and Meier, 1986, 1989; Leatherland et al., 1992; Hoque et al., 1998; Rahman et al., 2000; Takemura et al., 2004b). The contribution of such cues to the synchronization could not be determined in the present study, since natural photoperiod including moonlight, was provided in the aquarium. Further studies under constant conditions (no tide and/or no light conditions) will be necessary to determine whether the reproductive cycles are synchronized by tidal or moonlight cues.

The molecular and physiological mechanisms of the endogenous lunar-synchronized rhythm and its entrainment to environmental cues are largely unknown. To date, few studies have been conducted on the molecular regulation of lunar-related spawning rhythm. In rabbitfishes. which strictly repeat spawning with lunar cycle, melatonin has been shown to be important in mediating between the moonlight cycle and physiology (Rahman et al., 2004; Takemura et al., 2004a). Receptors for melatonin showed diurnal and circadian expression in the brain and retina of the golden rabbitfish (Park et al., 2007). In grass puffer, we previously showed that four subtypes of melatonin receptor genes were synchronously expressed in the diencephalon with diurnal and circadian variations (Ikegami et al., 2009). The moonlight cue might be involved in the entrainment of the endogenous semilunar rhythm through melatonin in grass puffer. Alternatively, hydrostatic pressure (tidal stimuli) has been shown to influence behavioral and physiological functions through monoamine concentrations in the brain of eels (Sebert et al., 1986) and flounder (Damasceno-Oliveira et al., 2006, 2007). Examination of the changes in the monoamine concentrations in the brain of grass puffer during the spawning period is therefore of considerable interest and importance.

In conclusion, grass puffer showed aggregation behavior for spawning in precise semilunar cycles in the aquarium without tidal changes. The present study suggests that the semilunar spawning rhythm is endogenously maintained, while tidal stimuli are important for the entrainment of the spawning rhythm. Grass puffer provides a useful model for the study of the molecular and physiological regulation of lunar-related spawning rhythm.

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