Female spawning strategy in *Rhinogobius* sp. OR: how do females deposit their eggs in the nest?

Noboru Okuda*✉, Sayaka Ito, and Hajime Iwao

Department of Biology and Earth Sciences, Ehime University, 2-5 Bunkyo-cho, Matsuyama 790-8577, Japan
** Present address: Center for Marine Environmental Studies, Ehime University, 3 Bunkyo-cho, Matsuyama 790-8577, Japan*
(e-mail: nokuda@sci.ehime-u.ac.jp)

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Abstract We tried to elucidate how females of a paternal nest brooding goby *Rhinogohius* sp. OR deposit their eggs in a nest, using a marking technique for live eggs under laboratory conditions in which male somatic condition, nest space, and mating pattern (monogamous or bigamous) were controlled. Whether females rejected mating was independent of either male quality, such as body size and somatic condition, or nest space. In a situation in which two females were allowed to spawn sequentially with a male, however, females rejected mating at a higher rate when they were the first to spawn than when they were the second to spawn; this is because eggs from first females were more vulnerable to cannibalism by parental males and second females. Even when nest space was limited and thus was occupied by eggs from the first females, second females could deposit all their eggs in the nest by using the minute interspace of existing eggs. In the presence of the parental male, such a female seemed less likely to suffer a cost from increased egg mortality due to crowdedness, still holding the advantage of being the second spawner. Finally, we extrapolated the field breeding ecology of this fish from the laboratory data. It was suggested that a single monolayer brood of the same age usually consisted of eggs from multiple females and thus the mating pattern would be more polygynous than previously expected.

Key words Cannibalism · Female counterstrategy · Egg marking · Mixed brood · *Rhinogobius*

In traditional ichthyology, field workers often focused on male behavior to study the nature of breeding ecology in fishes (Breder and Rosen, 1966). This preference is partly associated with the prevalence of conventional sex roles in fishes in which males are morphologically conspicuous and behaviorally vigorous while females are cryptic and coy (Darwin, 1871); thus, it is easier for field workers to locate and observe males than females in nature. However, the importance of female behavior has been recently emphasized and discussed to fully understand the reproductive nature of animals, especially in terms of behavioral ecology (Rosenqvist and Berglund, 1992; Ahnesjö et al., 1993).

To know when, where, and with whom females spawn under natural conditions is the first step in recognizing the presence of female mate choice and thus female reproductive strategy in fishes. A large number of field studies have reported female spawning timing (Johannes, 1978; Robertson, 1991), spawning site preference (Baylis, 1981), and mate preference (Andersson, 1994). However, we have potential difficulty in studying female strategy in paternal nest brooders, especially in those with a closed nest, because their spawning event is often hard to observe directly. Thus, we can only estimate the occurrence of female spawning from the resultant pattern of egg distribution in a focal nest, such as the number of eggs, variety of their developmental stages, and their spatial heterogeneity (i.e., the number of egg batches). With such a traditional method, this estimation greatly relies on the assumption that a female will deposit a discrete egg batch that can be discriminated from other egg batches.

The subject fish, *Rhinogobius* sp. OR (sensu Akihito et al., 2002), is a freshwater goby. Since the pioneer research by Mizuno (1960), the *Rhinogobius brunneus* species group is considered to consist of several undescribed species that are morphologically and genetically different (Masuda et al., 1989). *Rhinogobius* sp. OR originally inhabited the lacustrine environment but has recently extended its distribution over many river systems in Japan, due mainly to human introduction, which might be incidentally caused by fishery stocking with ayu, *Plecoglossus altivelis*, from Lake Biwa (Koshikawa, 1989). Kamo River in Ehime is one such river system. The early life history of this fish has been studied intensively (Yuma et al., 2000; Maruyama et at., 2001a,b), but there are no available data on adult biology, with particular reference to breeding ecology. In some types of the *R. brunneus* species group, breeding ecology has been examined by sample collection and field observation (Takahashi and Yanagisawa, 1999; Ito and Yanagisawa 2000; Takahashi, 2000; Takahashi et at., 2001). It is not yet well known, however, how females deposit their eggs in a nest.

In a companion paper (Okuda et al., 2002), we reported a marking technique for live eggs. Using this technique, we aimed to elucidate the female spawning strategy of *Rhinogobius* sp. OR in laboratory conditions under which ecological factors can be controlled.

Materials and Methods

Field collection.—We collected 20 nesting males of *Rhinogobius* sp. OR and their nest stones at Kamo River in the breeding season of 1997. After measuring total length (TL, mm) of these males, we released them at their capture sites. We also measured nest size (cm^2) represented as the peripheral area of nest stone, nest space (cm^2) potentially available for oviposition, and brood area (cm^2) represented as the total area of egg batches. The nest space was defined as the underside area of the nest stone from which nesting males wiped silt and algae. We scraped eggs off the nest and preserved them in 5% formalin to count the total number of eggs (i.e., brood size). In the laboratory, we sorted these eggs to three developmental stages (early, middle, and late) based on egg coloration and counted the number of live and dead eggs at each stage. From these measurements, we calculated the following variables: brood coverage (%) = 100 × (brood area)/(nest space), egg density (cm^{-2}) = (brood size)/(brood area), and egg mortality (%) = (the number of dead eggs)/(the total number of eggs).

Breeding conditions.—We captured *Rhinogobius* sp. OR at Kamo River before and during the breeding season of 2001. We sorted out small adults, which mainly consisted of 1-year-old fish, to minimize the effect of body size on reproductive strategy. We then sexed the fish on the basis of sexually dimorphic traits (male having longer dorsal fins, longer snout, and wider jaws) and assigned separately to stock tanks (see Okuda et al., 2002, for details of breeding conditions). We fed females on frozen bloodworm ad libitum twice a day. Males were assigned at random to two experimental groups: males in good and poor condition (hereafter, good males and poor males, respectively). Good males were fed on the same rations as that of females whereas poor males were fed once every 3 days (i.e., one-sixth of the rations of good males).

Experimental design.—We designed a three-way layout experiment manipulating the following factors: male somatic condition, sex ratio, and nest space. The male somatic condition was controlled by food rations, as already described. The sex ratio (male:female) was set to 1:1 or 1:2. We prepared two artificial nests (a 100-mm-diameter × 110-mm-long PVC pipe longitudinally cut into thirds), which differed from each other not in nest size but in nest space; this strategy enables us to remove any effects of the nest size on female mating preference, as in the case for some nest brooders, in which females prefer males with a larger nest (Bisazza et al., 1989; Takahashi and Kohda, 2002). To regulate the nest space, we covered the nest with a spiny vinyl mat on which females cannot deposit their eggs (Fig. 1). The small space nest (hereafter, small nest) had 36.0 cm^2 (6.0 × 6.0 cm) of space available for spawning. This space is nearly equal to the average area of a single egg batch ($\bar{x} \pm \mathrm{SD} = 36.3 \pm 14.6\,\mathrm{cm}^2$, $n = 6$) cared for by wild males of the small size class, which are expected to have mated with females as small as the subject females. For the large space nest (large nest), spawning space was 72.3 cm^2 (8.5 × 8.5 cm), which was twice that of the small nest. For each nest, we set an attachable translucent sheet on its spawning space, so that we could measure brood characteristics by the sheet on which females laid their eggs.

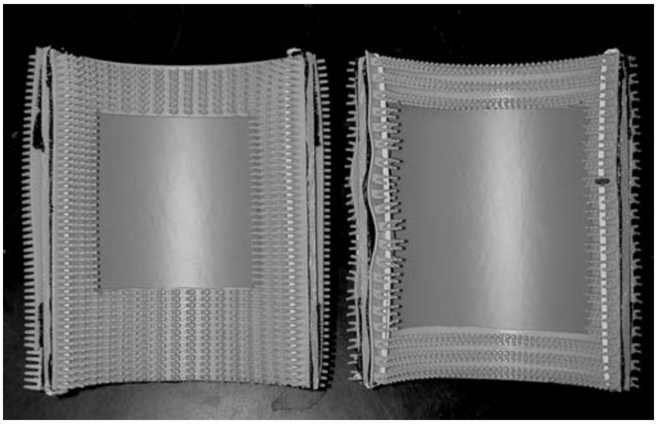

Fig. 1. Underside of nests covered with a spiny mat. Nest with large (*right*) and small (*left*) oviposition space

We conducted experiment from 31 May to 17 July. In the evening previous to each experiment, subject males that were selected randomly from stock tanks were introduced into an experimental tank (300 W × 450 L × 300 H) that was provided with a nest and gravel. At the same time, one or two mature females were also introduced into this tank. Until the onset of the experiment, each fish was isolated by compartments, but visual interactions were allowed between the sexes through a transparent board. In a bigamous situation, we prevented two females from visually contacting with each other, using an opaque board, in consideration of the possibility that the presence of a dominant female affects the decision for a subordinate female to spawn.

The experiment consisted of two stages (first and second), starting from early in the morning (at 0400–0500). In the first stage, experimental males were allowed to spawn with a first female for 6 h. This duration was long enough for females to complete spawning (Okuda et al., 2002). At the end of the first stage, we checked the presence of eggs in the nest. If the female did not spawn at all, this trial was considered a failure and discontinued. If the female did spawn, she was removed from the experimental tank. Subsequently, the mated male was allowed to spawn with a second female for 6 h more (second stage) in cases where the sex ratio was 1:2. Where the sex ratio was 1:1, a male was left alone in the tank for 6 h after removal of his first mate. At the end of the second stage, males and, if any, their second mates were removed from the tank.

With this experimental design, we expect that males spawning with two females in a small nest cannot receive all eggs, whereas males spawning with a female in a large nest have enough room for oviposition. When males mate with a female in a small nest or when males mate with two females in a large nest, the nest capacity will just match the egg supply.

After the experiment, we measured TL of subject fish. The fish were dissected and separated into liver, gonad, and eviscerated fish body parts. Eggs were sorted out from the stomach and gut contents. We dried and weighed these organs and eggs eaten to the nearest 0.1 mg. For each fish, we calculated the hepatosomatic index [HSI = 100 × liver weight (mg)/total body weight (mg)] and the condition factor [K = 10^3 × total body weight (mg)/TL (mm)] as indicators of somatic condition and gonadosomatic index [GSI = 100 × gonad weight (mg)/total body weight (mg)] as an indicator of gamete depletion. For each female, we also counted the number of mature eggs remaining in her ovary. The dry weight of eggs eaten was used as an indicator of cannibalism intensity. Subject fish involved in unsuccessful experiments were returned to the stock tanks without any measurements.

If spawning occurred, a translucent sheet with eggs was detached from the nest after the second stage. We photographed this sheet using a digital camera and then put it into an incubation tank (also see Okuda et al., 2002). Three days after incubation, the sheet was photographed again. From a digital image of the brood at the early developmental stage, we measured brood area (cm^2) and the number of eggs in each of five 1.0 × 1.0 cm quadrats set on the brood image. We calculated egg density (cm^{-2}) by taking an average of five samples and brood size by multiplying the brood area by the egg density. We also counted the number of dead eggs at both early and late developmental stages to calculate egg mortality (%).

Twelve replicates were conducted for each treatment, except for cases in which spawning resulted in a failure. In two cases where spawning succeeded, we missed data on brood characteristics because we failed to set a translucent sheet on the nest.

Marking eggs.—In some cases where two females spawn sequentially with a male, we used a marking technique for live eggs to see how each female deposited her eggs in a nest, which female ate more eggs, and whose eggs were eaten more. Brilliant blue FCF and β-carotene were used for marking eggs. Details of the marking procedure are described in a companion paper (Okuda et al., 2002).

Data analysis.—For data analysis, we used parametric tests when data fitted a normal distribution; otherwise, we used nonparametric tests. Egg mortality was arcsine-transformed and then log-transformed to fit a normal distribution. Means are given ±SD, for descriptive purposes, and all P values are two-tailed.

We performed multiple regression analysis to examine what affects the egg mortality at late stage, incorporating the following factors: TL, HSI and K of males, average TL of mates, sex ratio, nest area, egg density, brood size, and the number of dead eggs at the early stage. There were strong correlations between HSI and K ($r = 0.75$) and between egg density and brood size ($r = 0.84$). To reduce the effect of colinearity on the multiple regression analysis, we extracted a primary component of the HSI and K with a principal component analysis (PCA) and substituted it as an index of somatic condition. The egg density was replaced by its residuals against the brood size. We adopted full and reduced models, the latter being calculated by a stepwise method.

Results

Field data. Nest and brood characteristics of parental males from a Kamo River population are shown in Table 1. Some broods consisted of eggs at three different developmental stages. Within a brood, egg batches at different ages were discretely distributed, whereas each egg batch formed a monolayer in which eggs were uniformly distributed. Because dead eggs were found only at the early developmental stage, egg mortality was referred to as that at the early stage (Table 1).

Field data showed that larger males had a nest with larger space ($r = 0.51$, $n = 20$, $P = 0.02$). Among males, reproductive success, measured as brood size, correlated positively with their TL ($r = 0.63$, $n = 20$, $P = 0.003$) and positively with their nest space at a marginally significant level ($r = 0.42$, $n = 20$, $P = 0.06$). After substituting residuals of

Table 1. Nest and brood characteristics of parental males ($n = 20$) from a Kamo River population

Characters	χ	SD	Min	Max
TL of male (mm)	59.9	7.31	51.0	74.0
Nest area (cm^2)	126.0	61.1	38.9	285.7
Brood area (cm^2)	53.5	24.4	13.7	95.2
Brood coverage (%)	44.9	15.9	18.1	77.1
Number of egg batches at different developmental stages	1.65	0.81	1	3
Brood size	9141	3730	3320	15909
Egg density (cm^{-2})	181.9	43.7	117.5	275.9
Early egg mortality (%)[a]	0.87	−0.75 +3.78	0.08	12.03

[a] Sample size, $n = 8$

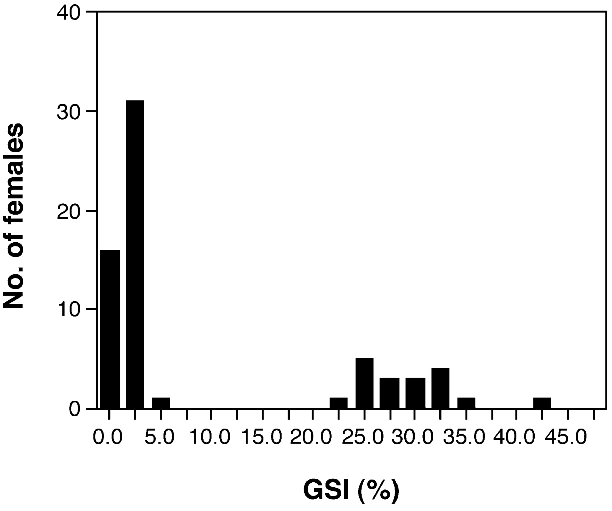

Fig. 2. Frequency distribution of gonadosomatic index (GSI) for second females

the nest space against the TL, a stepwise multiple regression analysis showed that the TL but not the nest space was a determinant of male reproductive success (TL: $\beta = 0.63$, $F = 11.94$; $R^2 = 0.40$, $n = 20$, $P < 0.003$).

Spawning success. Of 267 females that were provided in the first stage, 114 spawned in a nest. In cases without spawning, males always displayed nesting behavior and courted females vigorously, suggesting that a failure to spawn was not attributable to rejection by males. Spawning was successful regardless of whether nest space was small (56/114 = 49.1%) or large (58/153 = 37.9%; $\chi^2 = 3.36$, $df = 1$, $P = 0.07$) and whether the sex ratio was 1:1 (48/110 = 43.6%) or 1:2 (66/157 = 42.0%; $\chi^2 = 0.07$, $df = 1$, $P = 0.80$). Although good males were actually in better somatic condition (HSI: 17.4 ± 3.9%, K: 2.8 ± 0.3, $n = 57$) than poor males (HSI: 8.5 ± 2.9%, K: 2.3 ± 0.3, $n = 57$; *t* test, HSI: $t = -13.9$, $df = 112$, $P < 0.001$, K: $t = -9.0$, $df = 112$, $P < 0.001$), their somatic condition did not affect spawning success (good: 57/125 = 45.6%, poor: 57/142 = 40.1%; $\chi^2 = 0.81$, $df = 1$, $P = 0.37$).

In the sex ratio 1:2, it was uncertain whether second females contributed their eggs to the final brood unless their eggs were marked. To judge if the second females spawned, we examined their GSI and the number of mature eggs in their ovaries. Among 66 second females, GSI showed a discrete bimodal distribution (Fig. 2). All females with a low GSI had no mature eggs in their ovaries, except 1 female with 369 eggs in her ovary (GSI = 2.4%). Females with a high GSI, in contrast, had on average 4043 mature eggs (±1027, $n = 18$) in their ovaries. This number was equivalent to the number of eggs laid only by a female in the sex ratio 1:1 (3723 ± 1636, $n = 48$, *t* test, $t = 0.77$, $df = 64$, $P = 0.44$). Therefore, we regarded these 18 second females with high GSI as nonspawners. Based on this assumption, we estimated that 72.7% (48/66) of the second females spawned. This proportion was higher than that of first females that accepted mating (66/157 = 42.0%; $\chi^2 = 19.10$, $df = 1$, $P < 0.001$). Between cases in which the second spawning was successful or unsuccessful, there were no significant differences in either male body size (*t* test, $t = 1.19$, $df = 64$, $P = 0.24$), female body size ($t = 0.49$, $df = 64$, $P = 0.62$), sexual size difference ($t = 0.65$, $df = 64$, $P = 0.52$), or size difference between first and second females ($t = -0.05$, $df = 64$, $P = 0.96$). Second spawning success was also independent of either nest space (small: 24/32 = 75.0%, large: 24/34 = 70.6%; $\chi^2 = 0.16$, $df = 1$, $P = 0.69$) or male somatic condition (poor: 24/33 = 72.7%, good: 24/33 = 72.7%; $\chi^2 = 0.00$, $df = 1$, $P = 1.00$).

Brood characteristics. The effects of nest space and the number of spawning females on brood size, brood area, and egg density are shown in Fig. 3. Brood size was affected by the number of spawning females (two-way ANOVA, $F = 167.82$, $df = 1$, $P < 0.001$) but not by nest space ($F = 0.08$, $df = 1$, $P = 0.78$; Fig. 3A). The size of a brood from two females was about twice as large as that from one female. The brood area was affected both by the number of females ($F = 33.05$, $df = 1$, $P < 0.001$) and by the nest space ($F = 151.86$, $df = 1$, $P < 0.001$; Fig. 3B). When the nest space was small, the oviposition space was nearly covered with eggs from one female (98.4%) and was overcrowded by eggs from two females (121.2%). Such overcrowdedness resulted from females depositing their eggs outside the available space through a small slit between the PVC pipe and the spiny mat. When females spawned monogamously in a large nest, compared to a small nest, their clutch area was enlarged but there was still enough room for additional eggs (75.4%). The egg density became high as the number of spawning females increased ($F = 135.37$, $df = 1$, $P < 0.001$) and as the nest space was reduced ($F = 65.17$, $df = 1$, $P < 0.001$; Fig. 3C). There was also a significant interaction between the number of females and the nest space ($F = 7.39$, $df = 1$, $P < 0.008$). The egg density was 2.6 times higher when two females spawned in a small nest than when one female spawned in a large nest.

At early developmental stage, some eggs died of physical damage. In three cases, an entire egg batch was unfertilized because of unknown cause, so that these data were excluded from the following analysis. The early egg mortality was 0.14% (+0.47 SD, −0.12 SD, $n = 109$). This rate was irrespective of either the number of spawning females (two-way ANOVA, $F = 1.04$, $df = 1$, $P = 0.31$) or nest space ($F = 0.81$, $df = 1$, $P = 0.37$). Egg mortality increased at the late developmental stage (3.88%, +10.69 SD, −2.96 SD, $n = 109$), ranging from 0.10% to 89.87%. The main cause of late embryonic death was a fungus infection. We performed a multiple regression analysis to look at factors affecting the late egg mortality (Table 2). A reduced model showed that late egg mortality increased as an early brood was damaged more, egg density was higher, and female body size was smaller.

Between natural and laboratory conditions, brood characteristics were compared. An average brood area from a natural population was intermediate between the oviposition space of small and large artificial nests, whereas brood size was larger in the natural population (*t* test, $t = -5.76$, $df = 130$, $P < 0.001$). Egg density was also significantly higher in the natural than the laboratory conditions (*t* text,

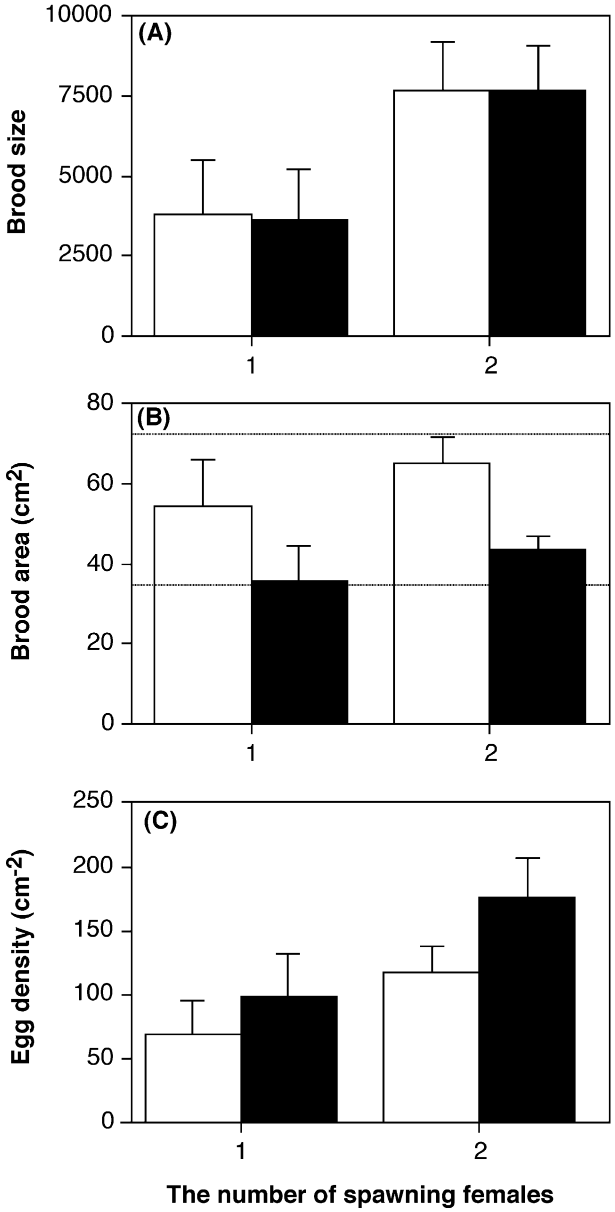

Fig. 3. Effects of nest space (small, *black*; large, *open*) and the number of spawning females (1 and 2) on brood size (total number of eggs) (**A**), brood area (cm^2) (**B**), and egg density (cm^{-2}) (**C**). Sample sizes are 34, 32, 23, and 23 for a large nest with a female, a small nest with a female, a large nest with two females, and a small nest with two females, respectively. In cases in which males failed to spawn with a second female in the sex ratio 1:2, the number of females is regarded as 1. Mean (*bars*) and SD (*vertical lines*) are given. *Horizontal dotted lines* indicate the maximum capacity for small (*lower line*) and large (*upper line*) nests

$t = -6.40$, $df = 130$, $P < 0.001$). Average egg density under natural conditions was 2.6 times higher than that in the laboratory condition under which one female spawned in a large nest, and was nearly equal to that when two females spawned in a small nest (Table 1, Fig. 3). Early egg mortality was higher in natural than laboratory conditions (*t* test, $t = -3.36$, $df = 115$, $P < 0.002$), although late egg mortality was negligible in the former.

Egg distribution. All broods took a monolayer form, often leaving small gaps that resulted from biting eggs. In ten cases in which we could discriminate between eggs from two females using egg markers, we viewed how two females deposited their eggs in a nest. In three cases (one for a large nest and two for a small nest), eggs from two females were discretely distributed (Fig. 4A). In these cases, the first female clumped her eggs in the center of the nest space and then the second female enveloped them in their eggs. In the other seven cases (five for a large nest and two for a small nest), the second female deposited each egg in the interspace of eggs laid by the first female, sometimes resulting in a homogeneously mixed brood (Fig. 4B,C).

Cannibalism. Females sometimes had eggs in their intestines at the end of the experiment. First females had empty intestines at a significantly higher rate (74/114 = 64.9%) than did second females (33/66 = 50.0%; $\chi^2 = 3.86$, $df = 1$, $P < 0.05$). When compared between two sequential females in the sex ratio 1:2, second females ate more eggs than did first females (Wilcoxon signed-rank test, $z = -2.03$, $P < 0.05$). The egg-marking technique revealed that second females did not preferentially cannibalize eggs of first females (Wilcoxon signed-rank test, $z = -0.71$, $P = 0.48$). For both first and second females, intensity of cannibalism was independent of their K and HSI (Spearman rank correlation, all $P > 0.05$).

Males cannibalized eggs much more (median = 8.80 mg, 25% percentiles = 4.35, 75% pereentiles = 16.15, $n = 114$), compared to females (median = 0.00 mg, 25% percentiles = 0.00, 75% percentiles = 3.50, $n = 180$; Mann–Whitney *U* test, $z = -10.0$, $P < 0.001$).

Discussion

Cannibalism. Female *Rhinogobius* sp. OR that were the first to spawn in a nest tended to infrequently eat eggs, in contrast with males, which cannibalized eggs frequently and in large quantities. In our laboratory, all egg eating by first females and males resulted in filial cannibalism. The current theory hypothesizes that filial cannibalism would evolve as an adaptive parental strategy when present reproductive loss caused by filial cannibalism could be offset by a future fitness benefit. This hypothesis is more likely applicable to males than to females because males can obtain an energetic advantage from egg cannibalism while making little gametic investment, as supported by a great number of empirical studies reporting that parental males often perform filial cannibalism in nature (Dominey and Blumer, 1984; Smith and Reay, 1991; FitzGerald and Whoriskey, 1992).

In species with maternal or biparental care, in contrast, females are rarely reported to perform filial cannibalism in natural conditions although sometimes can be incited to do so by experimental manipulation, such as brood reduction (Mrowka, 1987; Lavery and Keenleyside, 1990). Whenever females perform filial cannibalism, they incur a net energetic cost because expenditure in egg production can be only partly compensated by eating their own eggs, which

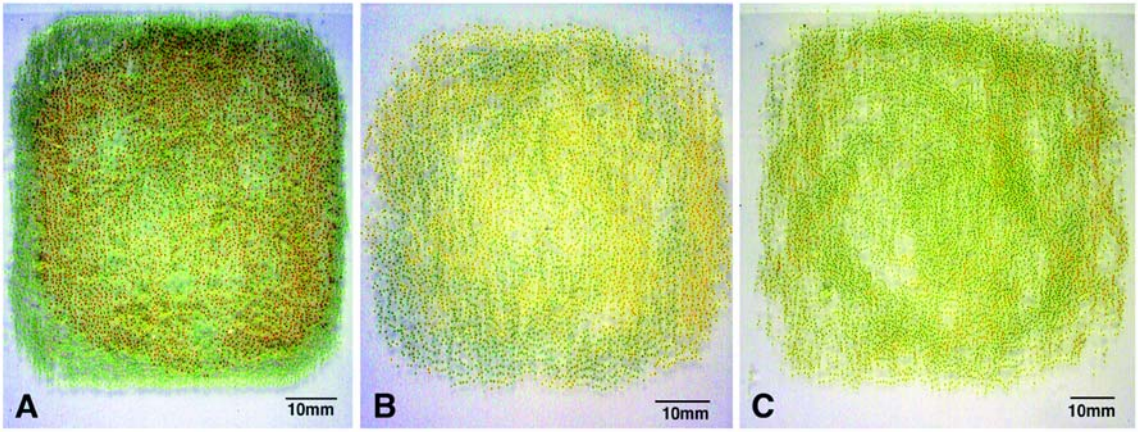

Fig. 4. Distribution of eggs from two females. **A** Discrete type. A batch of orange eggs (no treatment) from the first female is eveloped in green eggs (brilliant blue FCF treatment) from the second female. **B** Partially scattered type. Green eggs from the second female are partially clumped but scattered in the interspace of orange eggs from the first female. **C** Scattered type. Green eggs from the second female are scattered everywhere within a batch of orange eggs from the first female

Table 2. Factors affecting egg mortality at the late developmental stage

Variables	Full model			Reduced model		
	β	*t*	*P*	β	*t*	*P*
Male TL (mm)	−0.03	−0.33	0.74			
Male somatic condition	−0.08	−0.96	0.34			
Average TL (mm) of females	−0.08	−0.90	0.37	−0.16	−2.03	0.04
Sex ratio	0.11	1.10	0.27			
Nest area (cm^2)	−0.05	−0.33	0.74			
Egg density (cm^{-2})	0.22	1.55	0.12	0.28	3.54	0.001
Brood size	−0.19	−1.87	0.06			
Number of dead eggs at early developmental stage[a]	0.05	6.20	0.001	0.50	6.39	0.001
	$R^2 = 0.37, F = 7.54, P < 0.001$			$R^2 = 0.34, F = 18.45, P < 0.001$		

β, standardized partial regression coefficient

[a] Data were log-transformed

assimilation entails an energetic loss. Thus, conditions favoring filial cannibalism are more limited for females than for males.

In a situation where mating pattern is sequentially bigamous, second females cannibalized more eggs than did first females. The first females were purely filial cannibals, whereas the second females could be heterocannibals to some extent. Heterocannibalism by females is widespread among fishes (Dominey and Blumer, 1984; Smith and Reay, 1991). In paternal care species, heterocannibalism by females sometimes predominates over filial cannibalism by parental males (FitzGerald and Whoriskey, 1992). Also, in a natural population of *Rhinogobius* sp. LD (sensu Akihito et al., 2002), which is closely related to the subject fish of this study, females are dominant cannibals (Ito and Yanagisawa, 2000). Heterocannibalism is advantageous for females because it enables them to compensate for energy depletion due to spawning or energy needed to replenish new eggs, without sacrificing their own offspring (Meffe and Crump, 1987; Belles-Isles and FitzGerald, 1993). In our experiment, however, females were not heavily cannibalistic, compared to males. Because food rations were high for laboratory females, they might have had surplus energy reserves, suggested by their egg productivity, which was two to three times higher than that of wild females (Okuda et al., 2002).

Ito and Yanagisawa (2000) found, in *Rhinogobius* sp. LD, that females ate eggs laid by previous females before they spawned, and suggested that these females would do so to make room to deposit their own eggs. In our experiment, in contrast, second females appeared to perform cannibalism during or after their spawning because their intestines contained their own eggs as well as eggs of first females. In some fishes, it has been reported that parents can discriminate their own eggs from unrelated eggs (Loiselle, 1983; FitzGerald and van Havre, 1987; Smith and Whoriskey, 1988). In *Rhinogobius* sp. OR, females that have already laid their eggs are less likely to have the option to cannibalize unrelated eggs selectively, irrespective of their ability to recognize relatives. The selective cannibalism will be constrained by female spawning strategy, where females should deposit their eggs in the interspace of eggs laid by previous females if there is no enough space for oviposition (see following).

Female spawning strategy. Females showed no clear tendency to refuse mating selectively in relation to either male quality, such as body size and somatic condition, or nest space. However, females that were allowed to mate bigamously rejected mating at a higher rate when they were the first to spawn than when they were the second to spawn. We do not think that females might be inclined to spawn more frequently in the afternoon (i.e., second stage) than in the morning (i.e., first stage) because they show no clear diurnal spawning periodicity (S. Ito, personal observation). There also seems to be little possibility that dominant females suppress spawning by subordinate females (e.g., the pipefish *Syngnathus typhle*; Berglund, 1991), as suggested by our result that the size difference between bigamous females had no effects on spawning occurrence. Although our experiment was not designed to examine female mating preference explicitly, the following evidence suggests that unsuccessful spawning resulted from females rejecting an unwanted mating; all females that did not spawn had a fully mature ovary and some of them even released their eggs outside the nest without fertilization, in spite of vigorous male courtship displays. Therefore, unsuccessful spawning appears to reflect a decision for females to avoid depositing their eggs in a focal nest.

A possible reason why females avoided being the first to spawn in the empty nest is that a first clutch was more vulnerable to heterocannibalism by the subsequent spawners. Another is that males cannibalized more eggs from the first female when two females were available to them simultaneously (N. Okuda, et al., unpublished data). Whichever is more influential, it is obvious that a risk of egg cannibalism is higher for the first spawner than for the second spawner. In many nest brooders, females show a preference for males already having eggs in their nest (Unger and Sargent, 1988; Knapp and Sargent, 1989; Kraak and Groothuis, 1994; Forsgren et al., 1996; but see Jamieson and Colgan, 1989), which is explicable in terms of female counterstrategy against cannibalism (Lindström, 2000).

Although females can benefit from avoiding spawaning in a nest without eggs, they may risk losing an opportunity for oviposition if nest space is limited. Contrary to this prediction, our experiment revealed that even when a nest had been occupied by eggs from first females, second females could deposit all their eggs in the interspace of existing eggs; that is to say, they used a large number of minute oviposition spaces. On the other hand, such behavior caused an increase in egg mortality in the absence of parental males because crowding lowers the air supply for each egg. In addition, early physical damage to eggs, which could result from cannibalistic behavior such as biting, aggravated late egg mortality.

The second females might not suffer a large fitness cost from increased egg mortality, however, if parental males were present. In some paternal fishes, males increase parental investment with increasing clutch size (Coleman et al., 1985; Sargent, 1988); this is also true for *Rhinogobius* sp. OR in which males increased the frequency of fanning behavior as they received a second clutch (N. Okuda et al., unpublished data). Hence, the increased cost caused by sequential spawnings will be inflicted on males but not on females, taking the form of increased energy or time expenditure on parental care. The field data showed that the proportion of dead eggs was considerably larger at the early developmental stage but was reduced to null at the late stage, which suggests that increased risk of egg mortality due to crowding could be compensated by increased paternal care.

A series of our experimental results have an important implication for understanding the field breeding ecology of nest brooders. Our marking technique for live eggs revealed that a monolayer of eggs at the same age was laid by multiple females and varied greatly in egg density, with its peripheral area being unchanged. Traditional field studies on nest brooders have often used the number of discrete egg batches or their peripheral area as parameters to estimate the mating pattern and male reproductive success (Downhower and Brown, 1980; Itzkowitz and Makie, 1986; DeMartini, 1987; Côte and Hunte, 1989; Knapp and Warner, 1991). However, as suggested by Takahashi and Yanagisawa (1999) and Ito and Yanagisawa (2000), males of the *Rhinogobius brunneus* species group often spawn with multiple females simultaneously or sequentially within a day, resulting in a mixed brood consisting of eggs at the same age. From such a brood characteristic, one may judge that a focal male cares for eggs from one female in a breeding cycle and thus its mating pattern is monogamous. We emphasize here that these measurements are not always appropriate for estimation of breeding ecology without elucidating how females deposit their eggs.

Finally, we extrapolated the breeding ecology of *Rhinogobius* sp. OR in a natural population from the laboratory data. Assuming that 1 female lays 3723 eggs in a nest with unlimited space (i.e., large nest) with a density of 68.9 eggs/cm^2, we estimate that males, in nature, will have eggs from 1 to 4 females at a given time of breeding cycle and that a batch of eggs at the same age will be derived from 2.6 females on average. In this fish, therefore, the mating pattern is more polygynous than expected from traditional approaches.

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