A marking technique for live fish eggs and larvae

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Abstract A new marking technique for live fish eggs and larvae was proposed to elucidate the larval biology and adult breeding ecology of wild fish. In the laboratory, females of a freshwater goby *Rhinogobius* sp. OR were abdominally injected with one of three coloring agents—brilliant blue FCF, rose Bengal, or β-carotene—before their oviposition. The rose Bengal proved lethal to adult fish. The other two dyes had little effect on adult mortality. With these two treatments, there were negative effects on neither fecundity nor egg mortality, resulting in normally developed larvae. The brilliant blue FCF stained eggs and larvae greenish blue whereas the staining effect of β-carotene was unclear. The timing of injection was important in effective staining of eggs and reducing the risk of miscarriage. In conclusion, the brilliant blue FCF was the more useful marker. We discuss what this method can show us about the ecology of wild fish and how this method can be applied to field study.

Key words Egg mortality · Fecundity · *Rhinogobius* · Toxicity

In ichthyological studies, we often make anatomical observations on the early life stage of fishes by means of staining techniques to enhance visibility (Fukuhara and Tanaka, 1987). However, the toxicity of the staining reagent usually prevents us from keeping the fish alive in vivo or in vitro throughout their early life. If we can stain eggs and larvae without harming them, an increasing amount of knowledge will be accumulated on larval biology, which has puzzled many fish biologists. Some markers to stain the otolith have been proposed to elucidate larval biology (Tsukamoto, 1987). However, this technique does not allow identifying marked subjects by eye. Bartos and Wirtz (1984) developed a novel technique for marking live eggs and larvae visibly. They succeeded in staining eggs by providing the mother with dyed food in captive conditions. With this method, however, there was great intra- and interspecific variation in the staining effect, probably due to individual differences in food preference or metabolic rate. For this reason, the dietary treatment is not appropriate for marking eggs of only a focal individual or species in the field.

We here report a new method applicable to field study, using a freshwater goby *Rhinogobius* sp. OR (sensu Akihito et al., 2002) in which males care for eggs that the females lay on the underside of the stone nest. We discuss how this method can be used to elucidate the larval biology and adult breeding ecology in nature. We also suggest what attention should be paid to apply this method to the field study.

Materials and methods

Breeding condition.—We collected *Rhinogobius* sp. OR at Kamo River, Shikoku Island, Japan, before and during the breeding season of 2001. We used small adults, which mainly consisted of 1-year-old fish, to minimize the effect of body size on experimental results. After taking the fish to the laboratory, we sexed them and assigned separately to stock tanks (300 W × 600 L × 360 H mm). Fish density (the number of fish per tank) was held less than 34 for females and less than 21 for males. We equipped these tanks with gravel, especially male tanks with a pile of corrugated plastic sheet to mitigate agonistic interactions among males. We kept laboratory conditions constant as far as possible (water temperature: $\bar{x} \pm SD = 21.1 \pm 0.3$°C, range = 20.3–22.0°C; light regimen: 14 L : 10 D). Subject females were fed on frozen bloodworm twice a day.

Marking procedure.—We prepared three kinds of reagents different in color for marking live eggs of this fish. We used brilliant blue FCF (blue no.1), rose Bengal (red no.105), and β-carotene, which are generally known as artificial coloring agents for food. We dissolved the former two markers in Locke's salinity solution (0.75% NaCl, 0.02% KCl, 0.02% $CaCl_2$, 0.002% $NaHCO_3$, and 0.1% $C_6H_{12}O_6$) to make a 1% solution. Because β-carotene is insoluble in water, it was dissolved in 100% ethanol and then diluted with Rocke's solution to provide a 0.5% β-carotene/1% ethanol solution. As a preliminary test, we injected 0.05, 0.1, and 0.2 ml brilliant blue FCF or rose Bengal into the abdominal cavity of each female through her cloaca, using a syringe with a small needle (27 G × 3/4 in.). The greatest care was taken not to inject the

markers into the ovary. These six subject females were allowed to breed freely throughout the experimental period unless they died. Three subjects with brilliant blue FCF survived to the end of the experimental period, whereas the three fish receiving rose Bengal all died the day following injection. Thus, we discontinued use of rose Bengal for this experiment.

In preparation for the experiment, we injected 140 females with 0.05 ml brilliant blue FCF solution and 27 females with 0.05 ml β-carotene solution. A dosage was equivalent to 0.0032% of dry fish body weight for brilliant blue FCF and to 0.0014% for β-carotene. The brilliant blue FCF was injected one to three times per capita during the entire experimental period whereas β-carotene was injected only once for all subjects. The other females that were given no treatment were regarded as a control. For convenience, females with brilliant blue FCF, β-carotene, and no treatment are hereafter referred to as blue females, red females, and control females, respectively.

Experimental procedure.—We conducted experiments 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). At the same time, females that were judged mature from their nuptial coloration and belly expansion were also introduced into either of two female's compartments at random. In experimental tanks, subject females were allowed to spawn with a nesting male monogamously (sex ratio 1:1) or bigamously together with another female in sequential order (sex ratio 1:2). The latter experiment was conducted to ascertain whether it is possible to visually discriminate between eggs from two females when one of them is marked. Each female was allowed to mate for 6 h, which was long enough to complete spawning, as demonstrated by a preliminary experiment in which females could finish spawning within 3 h ($n = 3$). Further details of the experimental procedure are described in a companion paper (Okuda et al., 2002).

Before the experiment, we set an attachable translucent sheet on the ceiling of an artificial nest (a 100-mm-diameter × 110-mm-long PVC pipe longitudinally cut in thirds) and allowed females to deposit their eggs on this sheet. After the experiment, we detached it from the nest to photograph the brood on the sheet using a digital camera. We then put it into an incubation tank (170 W × 300 L × 240 H mm) that was weakly aerated and sterilized by a low concentration of methylene blue. Three days after incubation, it was photographed again. Based on a digital image of the brood, we counted the number of live and dead eggs both at early (day 0) and late (day 3) developmental stages to calculate egg mortality (%), the proportion of dead eggs to total eggs. The egg mortality was arcsine-transformed and log-transformed to normalize its distribution. Three cases in which an entire clutch was unfertilized were excluded from the analysis.

In the sex ratio 1:1, we obtained 13 broods from blue females, 4 from red females, and 31 from control females. In the sex ratio 1:2, we also obtained 36 mixed broods from blue and control females, 5 from red and control females, and 25 from two control females. In 18 of these cases, the second female did not spawn.

Results

Adult mortality and fecundity. Soon after injection of brilliant blue FCF, fish body coloration became vivid blue and thereafter faded away gradually, returning to the normal coloration within a few weeks. β-Carotene made the body coloration slightly reddish for a few days. Of 140 blue females, 128 survived over the entire experimental period unless they were used for an experiment. Their mortality (8.57%) was not particularly serious, although it was significantly higher than that for control females (3/159 = 1.89%; $\chi^2 = 6.98$, $df = 1$, $P < 0.009$). By contrast, no red females died (0/27 = 0.00%).

In cases in which females spawned monogamously with a male, their fecundity (i.e., the number of eggs) did not differ significantly among treatments (red: $\bar{x} \pm$ SD = 4567 ± 1836, $n = 4$; blue: 3106 ± 1651, $n = 18$; control: 3880 ± 1607, $n = 44$; ANOVA, $F = 2.03$, $df = 2$, $P = 0.14$). However, it is premature to conclude that the β-carotene did not have any effects on female fecundity (control vs. red; a post hoc Bonferroni–Dunn method, $P = 0.09$), because of its small sample size.

Each female that was injected with 0.05, 0.1, or 0.2 ml of brilliant blue FCF spawned five, six, or four times during the entire experimental period, respectively.

Staining effect and egg mortality. Brilliant blue FCF stained the yolk of eggs greenish-blue, and the coloration remained at least until the larval stage. With β-carotene, on the other hand, eggs were stained slightly reddish but were not markedly discriminable from the normal yellow eggs. The former was therefore effective in discriminating between eggs from two females within a mixed brood (Fig. 1A). Subject females spawned at a variety of intervals after injection with brilliant blue FCF. In cases where females spawned within 8 days of injection, their eggs were stained more effectively than those of females injected more than 8 days before spawning (Table 1; the probability that the staining effect was at least detectable, Fisher's $P < 0.001$). However, when we injected fully mature females, they often released their eggs without fertilization in the stock tanks. For three blue females that were allowed to breed repeatedly, eggs from a second clutch were no longer bluish, suggesting that this marker could be effectively taken into the egg yolk during the process of egg production; otherwise, it would be gradually catabolized. For only two of eight red females spawned was the staining effect detectable. In these two cases, one spawned 1–4 days after injection and another 7–14 days after (the date of injection could not be exactly identified for each individual).

Under both treatments, eggs normally developed (Fig. 1B) and newly hatched larvae swam vigorously (Fig. 1C). The egg mortality was not significantly different among treatments (control: 2.96% + 8.24 SD, –2.27 SD, $n = 42$; brilliant blue FCF: 4.84% + 10.14 SD, –3.39 SD, $n = 18$; β-carotene: 2.57% + 7.59 SD, –2.00 SD, $n = 4$; ANOVA, $F = 0.95$, $df = 2$,

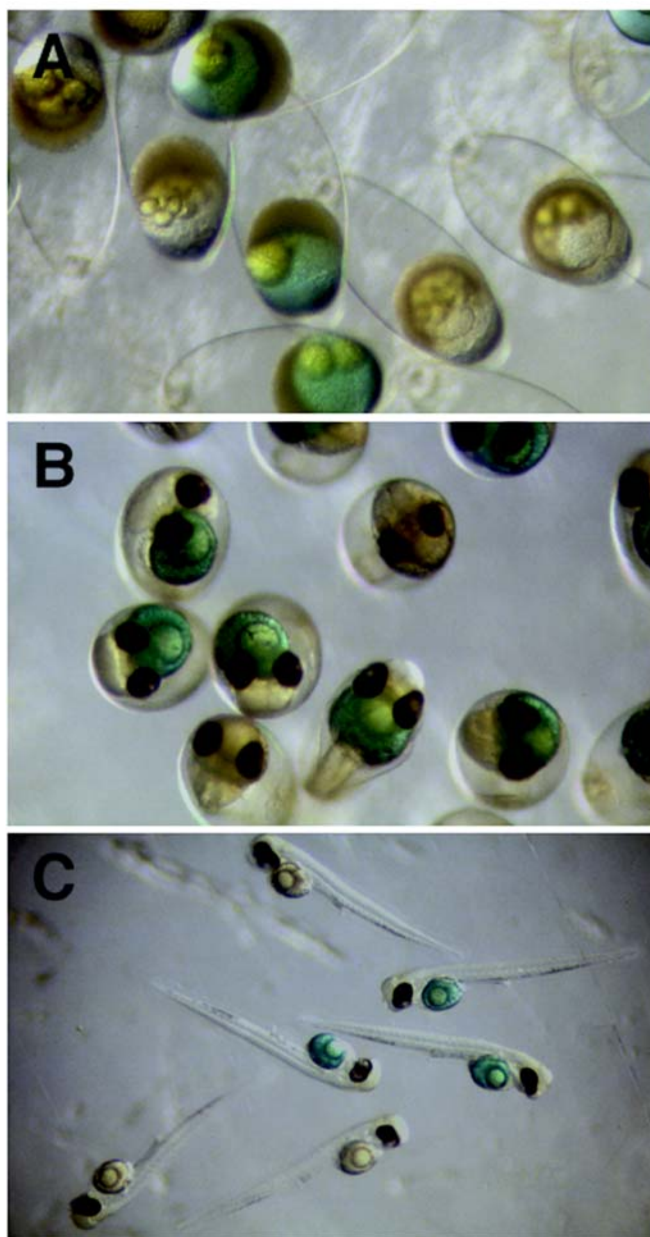

Fig. 1. A mixed brood from blue females (*blue eggs*) and red females (*yellow eggs*). In this case, the latter eggs were not markedly stained and thus their coloration was similar to normal. Eggs at early developmental stage (day 0) (**A**), at late developmental stage (day 3) (**B**), and newly hatched larvae (day 3) (**C**)

Table 1. The effects of injection timing (the latency period until spawning) on staining eggs with brilliant blue FCF

	n	1–8 days	>9 days	Unknown[a]
Clearly detectable	13	10	3	0
Slightly detectable	10	5	2	3
Undetected	19	0	13	6

[a] Cases in which we could not identify when subjects were injected

$P = 0.39$). Such a tendency remained unchanged after including cases in which eggs from either of two females was marked (ANOVA, $F = 0.15$, $df = 2$, $P = 0.86$).

Discussion

The present study revealed that β-carotene had no negative effect on adult mortality, whereas treatment with brilliant blue FCF caused a slight but significant increase in mortality. However, this result does not necessarily mean that the latter was noxious to adult fish per se. Blue females were injected repeatedly, in contrast to red females, all of which were injected only one time. At the beginning of the experiment, injected females sometimes suffered from a fungus infection in their abdomens; when they wriggled out of our hands, their viscera might have been scratched by a needle, due to our lack of experience. These accidental injuries became less frequent as our experience increased. In this study, we did not wish to use anesthesia to avoid its effect on fish mortality. However, we recommend that fish should be tranquilized with a very low concentration of an anesthesic agent to improve mortality. The use of a disinfected needle is also desirable.

Three blue females that were allowed to breed freely spawned much more frequently than wild females that spawned about twice in a season (S. Ito, personal observation). Although this result may be in part ascribed to the high level of rations available in the laboratory, it is less likely that brilliant blue FCF has a negative effect on female fecundity.

We conclude that brilliant blue FCF is a more useful marker than β-carotene because the latter was harmless but its staining effect was unclear. Because we cannot eliminate the possibility that β-carotene, a kind of carotenoid, has any positive effects on fecundity or egg quality, as reported for some fish species (Olson and Owens, 1998), future research should not be designed to compare these two markers. We suggest that a control group should be injected only with Locke's solution rather than be given no treatment, taking the effect of the injection itself into consideration.

Using this method, we expect to gain much information on the ecology of wild fish. For example, we can see when, where, and how a focal female deposits her eggs in nature. If the female provides parental care, does she care for only young that have a parental relation with her, and to what extent are her offspring superior or inferior to others in terms of survival and competitive ability? In species in which males care for eggs from multiple females, how many mates do they get in a breeding cycle and whose eggs are cannibalized by whom? To answer these questions will lead to better understanding of the breeding ecology in fish.

Finally, we suggest what is needed to apply this method to field study. First, we need to determine the best timing of injection for subject species. Our result suggested that abdominal injection during the maturation period might increase the risk of miscarriage. Before injection, we should therefore look at any sign of premature status for subject females, such as changes in nuptial coloration and belly

expansion, and should not use fully mature individuals but those nearly mature. In addition, subjects should be kept in the cage for a few days after injection because they become conspicuous to potential predators because of the change in their color. This problem is very critical for species that are cryptic and in great danger of predation.

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