Video Article

Basic Methods for the Study of Reproductive Ecology of Fish in Aquaria

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Abstract

Captive-rearing observations are valuable for revealing aspects of fish behavior and ecology when continuous field investigations are impossible. Here, a series of basic techniques are described to enable observations of the reproductive behavior of a wild-caught gobiid fish, as a model, kept in an aquarium. The method focuses on three steps: collection, transport, and observations of reproductive ecology of a substrate spawner. Essential aspects of live fish collection and transport are (1) preventing injury to the fish, and (2) careful acclimation to the aquarium. Preventing harm through injuries such as scratches or a sudden change of water pressure is imperative when collecting live fish, as any physical damage is likely to negatively affect the survival and later behavior of the fish. Careful acclimation to aquaria decreases the incidence death and mitigates the shock of transport. Observations during captive rearing include (1) the identification of individual fish and (2) monitoring spawned eggs without negative effects to the fish or eggs, thereby enabling detailed investigation of the study species' reproductive ecology. The subcutaneous injection of a visible implant elastomer (VIE) tag is a precise method for the subsequent identification of individual fish, and it can be used with a wide size range of fish, with minimal influence on their survival and behavior. If the study species is a substrate spawner that deposits adhesive eggs, an artificial nest site constructed from polyvinyl chloride (PVC) pipe with the addition of a removable waterproof sheet will facilitate counting and monitoring the eggs, lessening the investigator's influence on the nest-holding and egg-guarding behavior of the fish. Although this basic method entails techniques that are seldom mentioned in detail in research articles, they are fundamental for undertaking experiments that require the captive rearing of a wild fish.

Video Link

The video component of this article can be found at https://www.jove.com/video/55964/

Introduction

Spectacular adaptive evolution is evident in the morphology, ecology and behavior of fishes[1]. Especially, ecological features relating to reproduction are especially diverse, and most of these can be directly influenced by individual fitness[2]. To gain insight into selective pressures that have led to the evolution of unique features in different fish species, direct observation of reproductive and social behaviors using live fish is often beneficial to substantiate theoretical hypotheses.

However, continuous field observations of fish may require specialized underwater equipment and facilities that are difficult to maintain. In these cases, observations of wild-caught aquarium-reared fish can be helpful[3,4,5]. In addition, efficient observations of fish behaviors that are otherwise rare or difficult to observe under natural conditions can become possible by manipulating experiments in aquaria[6,7,8]. Rearing fish under good conditions by minimizing artificial stress and physical damage is critical for accurate ecological investigations.

The pygmy goby *Trimma marinae* reaches 23-25 mm total length and is distributed in the western Pacific Ocean, where it is found in quiet, sheltered bays, at depths of 9-26 m[9]. In this work, *T. marinae* is used as a model to describe a series of basic techniques for the collection of fish using the self-contained underwater breathing apparatus (SCUBA), fish transport, and eventual acclimation of the fish to aquaria for direct observation of the study species' reproductive behavior and ecology.

Protocol

1. Collecting and Transporting Live Fish

NOTE: This protocol describes how to collect fish that possess a gas bladder, from a depth of ≥15 m to the surface. Rapid conveyance to the surface will induce expansion of the gas bladder by a change of pressure, which can seriously harm or kill the fish. Caution is warranted, as damage caused to the fish during this first step will negatively effect their survival and later behavior.

1. Prior to diving with SCUBA, gather the following materials: a hand net suitable for capturing the targeted species underwater; double polyethylene bags that are large enough for the fish; rubberbands (ø 80 mm x 6 mm); an oxygen cylinder for inflating the collection bags

at the surface; the Elbagin, which is an antimicrobial comprises 10% sodium nifurstyrenate; a polystyrene foam box in which to place the collection bags; pipettes; and, if necessary (refer to step 1.4), a rope with a length equivalent to maximum diving depth, as well as a weight of at least 2 kg.
2. Collect the targeted fish species using a hand net while diving; while still underwater, place the captured fish in a polyethylene bag and tie the mouth of the bag with a rubberband. If it will take ≥1 day to transport the fish to an aquarium, also preserve water from the natural habitat in other bags, to be used for renewing the fish-keeping water.
NOTE: For safety, the underwater tasks must be worked by at least two people.
3. Surface from the collecting point with the bags of fish at a speed of no more than 1 m/min: while at a depth of 10 m or more, stop ascending for 1 - 5 min every 2 m; and from a depth of 10 m to the surface stop for 1 - 5 min every 1 m. When the captured fish are unable to maintain their buoyancy in the bag (*i.e.*, if they float up with an expanded gas bladder while still trying to swim toward the bottom), either maintain same depth for 1 - 5 min or descend to 1 - 2 m deeper. Once the fish seem to recover their buoyancy, resume ascent to the surface.
NOTE: If there is a possibility that the air in SCUBA tank will run out while ascending, fasten a rope to the polyethylene bag and attach a ≥2 kg weight; after underwater operator has safely ascended, pull this up to the surface at the same speeds stipulated above.
4. At beach or boat, dissolve 10 ppm Elbagin in the water of each polyethylene bag after surfacing.
NOTE: Although some fish may not be able maintain their buoyancy just after being brought to the surface, most can be expected to recover within one day.
5. If the density of fish in a collecting bag is high, divide the fish among more bags to prevent from them damaging each other by rubbing against each other during transport. If the fish is an aggressive species, divide them individually between bags.
6. Inflate the collection bag with oxygen and again seal the mouth of the bag using rubberbands. When introducing the oxygen into the bag, lower the nozzle into the bag's water to increase the dissolved oxygen content; the fully expanded bag should be 1/4 full with water.
7. Keep the collection bags with fish in a polystyrene foam box, to maintain a stable water temperature and decrease the fish's stress under dark conditions.
8. If it will take ≥1 day to transport the fish to an aquarium, once per day exchange 1/4 to 1/3 of the water in each fish-keeping bag, using water retained from the habitat to which has been added 10 ppm Elbagin, and repack each with oxygen. Each day, remove dead fish and excreta on the bottom by hand net or pipette.
NOTE: If airplane transportation is involved, wait at least 1 day to acclimating fish to the air-pressure on surface before transportation because two steps pressure change (from underwater to surface, and surface to upper air) in the short term may be negatively affect the survival of the fish.

2. Acclimating the Fish to an Aquarium

1. Float the bag containing fish in an aquarium for 30 min to equalize the water temperature.
2. Over a 10 min period, incrementally exchange the bag water with aquarium water, to avoid shock caused by a sudden difference in water chemistry (*e.g.*, pH, salinity).
NOTE: A fish that becomes shocked by too great a difference in water chemistry may display an abnormal change in body color and/or behavior. Monitor this carefully during acclimation.
3. Dissolve 10 ppm Elbagin in the aquarium water.
4. Thereafter, once a day for 3 days, renew 1/3 of the aquarium water, having added 10 ppm Elbagin to the replacement water.
5. Finally, begin to eliminate the Elbagin by exchanging half the aquarium water once a day, until the color fades.

3. Injecting a Visible Implant Elastomer (VIE) Tag to Identify Individual Fish

NOTE: In this work, individual fish are identified using VIE tags; for examples, refer to Frederick[10], Olsen and Vøllestad[11], and Leblanc and Noakes[12]. Also, if the study species is large enough to hold in a hand, the surgical table used in step 3.2 will not be necessary.

1. Determine the injection position and tag color for each individual. The safest option is to choose an injection point in the thick muscular dorsal or caudal part of the body, and to avoid injecting it into the abdomen where internal organs may be pierced.
NOTE: A numbering system is described in **Figure 1**: if the study species is large enough for injection into 8 possible positions, this system will allow the identification of 154 individuals using a single color. Ten colors of VIE tags are available commercially. Choose the distinguishable color based on the body color of fish.
2. **For fish that are smaller than can be held in the hand, prepare a surgical table as follows (refer also to Kinkel *et al.*[13]) (Figure 2).**
    1. Cut out a soft sponge measuring 5 cm L x 5 cm W, and which is at least 5 - 10 mm lower than the height of a Petri dish.
    2. Cut a groove in the sponge of approximately 5 - 10 mm depth, and adjust its width to that of the approximate body width of the fish. Cut the polyvinyl chloride (PVC) board (0.3 mm thickness) to 5 cm L x 5 cm W, and bend it into a valley-fold (or M-shape).
    3. Set the grooved PVC board on top of the grooved sponge and then set the sponge into the Petri dish (ø 160 mm, 30 mm depth). Use water from the recovery tank to fill the Petri dish until the PVC board is adequately immersed.
3. Prepare the VIE tags according to the VIE tagging manual. Mix the elastomer materials and add the mixture to the 3-mL syringe with 29-gauge needle, as contained in the kit.
4. Prepare two water tanks to use while performing the tag injections: one for anesthesia and one for recovery; adjust the temperature and salinity concentration of these to match the water in the rearing aquarium. Use an air pump with an air stone to mildly circulate the water in the recovery tank.
5. Prepare an anesthesia liquid by mixing 2-methylquinoline with an equal volume of 99.5% ethanol; add this to the anesthesia tank to achieve a concentration of 18 ppm.
NOTE: The optimum concentration of the anesthesia liquid will depend on the species and body size of the individuals. Therefore, examine the optimum concentration for the study species in advance.
6. Transfer individual fish to the anesthesia tank and allow them to become anesthetized: that is, wait until fish do not react to being touched or to water vibrations when the outside of the tank is tapped. As the body color changes when it is anesthetized in many fish, also monitor the

body color carefully and judge whether fish is anesthetized from its changes. Because there is some possibility that fish may jump out of the anesthesia tank, keep it covered using a transparent acrylic board, at least until the fish begin to move slowly.
NOTE: Fine-tune the time needed to anesthetize and/or adjust the concentration of anesthesia liquid, depending on the study species and its body size. If the operculum movement is ever stopped by the anesthesia, the fish will be at a high risk of death.
7. As fish will weaken if their body is warmed, keep fingers in the relatively cold water while anesthetizing the fish.
8. Lift a fish from the anesthesia tank and quickly measure it or record the necessary data (total length, sex, *etc.*). If a fish appears to recover during measurement, immediately transfer it back to the anesthesia tank.
9. Transfer the fish to a surgical table. Set the fish ventral side down in the PVC groove. If the body size is very small, use a binocular microscope while making the injection. If the study species is large enough to hold in a hand, inject the VIE tag while it is being held.
10. Position the beveled side of the needle toward the outside and pointed toward the head of the fish. Insert the needle subcutaneously, more or less parallel to the body, and as near to just under the skin as possible. Adjust the insertion depth depending on the fish body size and the easiness to ultimately see the tag.
11. Inject VIE tag while withdrawing the needle, and stop the injection before the needle bevel reaches the needle entry point (this will be easy to discern if injecting the tag in a relatively wide area).
12. On completing the tag injection, immediately transfer the fish to the recovery tank. If recovery appears too slow, gently circulate the water by hand.
13. After recovery, return the fish to the rearing aquarium, and continue to add 10 ppm Elbagin to the water for 3 days.
NOTE: Under low-visibility conditions for VIE tags, UVA filtered light will facilitate recognizing the tags.

4. Counting the Demersal Adhesive Eggs

1. To create an artificial spawning nest to which the fish can deposit adhesive eggs, cut an opaque PVC pipe (ø 5 cm, 6 cm long) in half, perpendicular to the diameter.
NOTE: The above pipe size is suitable for spawning the relatively small goby *T. marinae*. Thus, adjust the PVC pipe size and form as is appropriate for the study species.
2. Print a 5 mm x 5 mm grid on a waterproof sheet and cut it out to fit the inside area of the PVC pipe.
3. Fix the waterproof sheet to the inside of the PVC pipe using a rubberband.
NOTE: If it is difficult for females to attach eggs on the waterproof sheet, and eggs fall from the sheet, make the sheet textured with sandpaper.
4. Cover the aquarium bottom with a layer of sand, 1 - 2 cm thick. Insert approximately one-third of the PVC pipe obliquely into the sand, with the sheet-fixed side down.
5. **After successful spawning by the fish, remove the sheet with the egg mass from the PVC pipe and place it in a Petri dish with aquarium water. Fix a new sheet inside the PVC pipe and place this back on the bottom of the aquarium. Finally, count the eggs by photographing the egg mass.**
    1. Alternatively, if observations of parental egg care are to be undertaken after spawning, be careful not to bring the eggs out of the water. Rather, use a Petri dish to scoop up the eggs and sheet along with aquarium water, photograph the egg mass quickly, and then carefully reaffix the sheet with the egg mass back into the same PVC pipe before returning it all to its original position on the bottom of the aquarium. Finally, count the eggs using the picture.

6. **Count the eggs using ImageJ (Figure 3). These automatic and manual cell counting methods are explained in detail in the following: Basic cell counting (The University of Chicago, Integrated Light Microscopy Core Facility, http://digital.bsd.uchicago.edu/image_j.php); Particle Analysis (http://imagej.net/Particle_Analysis); Cell Counter (https://imagej.net/Cell_Counter).**
    1. Otherwise, if eggs are deposited densely, estimate the number of eggs by calculating the area-density ratio: count eggs in a printed 5 x 5 mm^2 grid to calculate the egg density, measure the surface area that is covered with eggs by ImageJ, and estimate the total number of eggs from above density per unit and surface area. The above surface area measurement method by ImageJ is explained in the following: Set scale… (Research Services Branch, https://imagej.nih.gov/ij/docs/menus/analyze.html); it explains how to define the spatial scale of the image; Using ImageJ to Measure Surface Area (Keene State college, Academic Technology, https://dept.keene.edu/at/2011/04/15/by-matthew-ragan/).
    NOTE: If eggs are transparent, the black waterproof sheet is more suitable for the automatic counting method because ImageJ can recognize the outline of eggs individually.

Representative Results

Following the above methods, 41, 15 and 96 individuals of *T. marinae* were collected in April 2014, 2015 and 2016, respectively, offshore of Amami Oshima, Kagoshima Prefecture, Japan (**Table 1**). In each case, 25 (61%), 14 (93%) and 91 (95%) individuals lived until depositing eggs in an aquarium. As reported in Fukuda *et al.*[3], only one fish died before the end of the observation period in 2014, and fish spawning otherwise appeared to commence 7 days after captured, showing that the individuals were being reared under good conditions.

The VIE tags were visible and allowed identification of the individuals, even in this small-sized fish (**Figure 4A**, **4B**). A photograph of eggs deposited on a waterproof sheet is shown in **Figure 4C**, proving that they were visible enough to be counted. After it was removed and photographed, the sheet was returned to its former place in the aquarium, and the nesting male immediately continued paternal care (**Figure 4D**).

Together, these techniques can be employed in experiments aimed to record the social interactions and the reproductive success of all individual fish reared. Specifically, Fukuda *et al.*[3] investigated the reproductive ecology of *T. marinae* in aquaria using the above methods. The results demonstrated a positive correlation between female fecundity and female body size (**Figure 5**), whereas no difference in reproductive success was observed among differently size males (**Figure 6**). Also, *T. marinae* tended to establish a continuous reproductive pair and most spawning occurred within these pairs (**Table 2**). Only male *T. marinae* were observed to perform paternal egg care. The aggressive interaction between individuals suggested that the monogamous mating system may result from mainly female mate guarding.

Collection day	Collection depth	Collection method	Number of collected individuals	Stay duration	Transport day	Number of dead individuals			Survival rate
						Surfacing	Transport	Acclimation	
15 April 2014	- 21 m	Surface with fish until surface.	41	over night	16 April 2014	16	0	0	61 %
23 April 2015	- 19 m	Surface with fish until 12 m depth, pull up by rope until surface.	15	1 day	25 April 2015	1	0	0	93 %
26 April 2016	- 21 m	Surface with fish until 15 m depth, pull up by rope until surface.	96	1 day	28 April 2016	4	1	0	95 %

Table 1: Collection Conditions and Fish Survival Rate. Number of dead individuals indicates when and how many individual fish died.

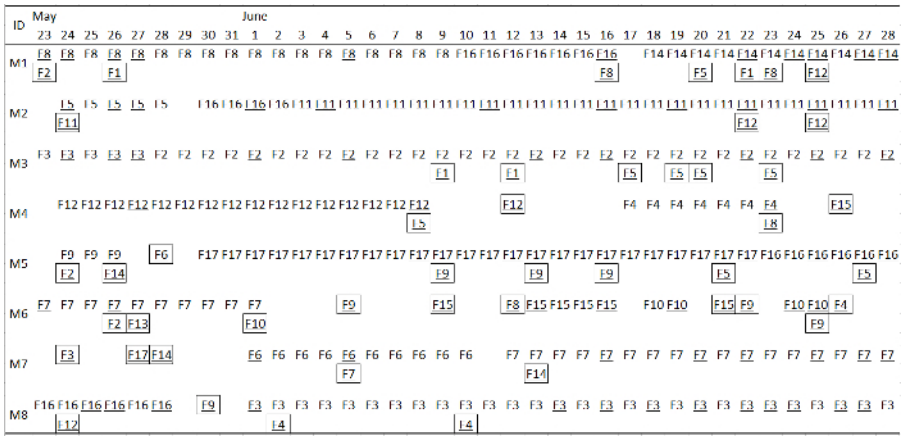

Table 2: Spawning Pairs during the Rearing Experiment. *M*, individual identity (ID) of males; *F*, ID of females; *underlined ID*, a spawned female; *outlined ID*, a female that mated with a male although they did not establish a continuous pair. These results indicate that *T. marinae* tend to establish a continuous reproductive pair as part of a monogamous mating system. This table has been modified from Fukuda *et al.*[3] Please click here to download this table.

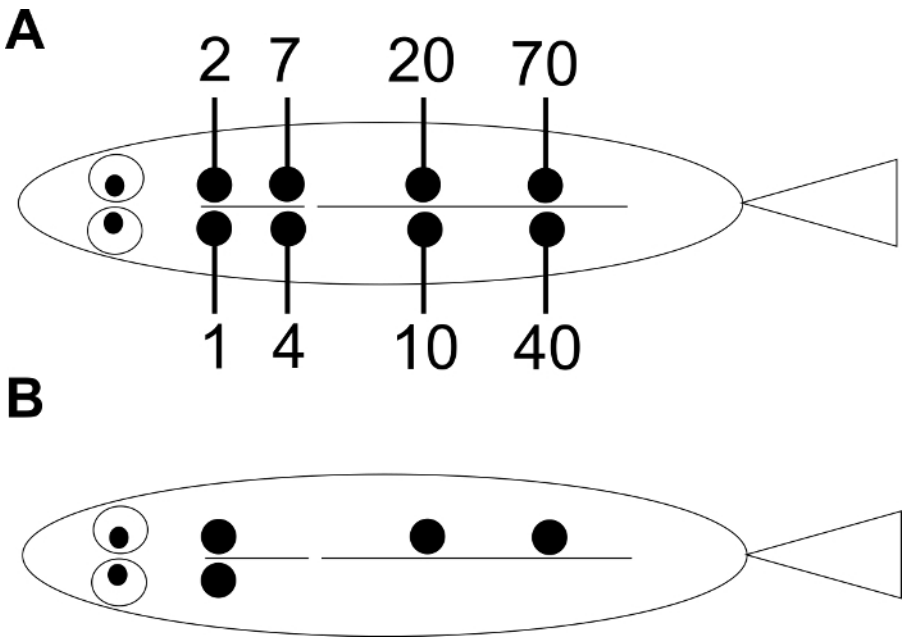

Figure 1: Arrangement of the VIE Tagging Positions. (**A**) Each numeral shows the number corresponding to the position of the injection. An individual fish's identity number is determined by matching up the position(s) of the tags. (**B**) An example of the individual fish No. 93.

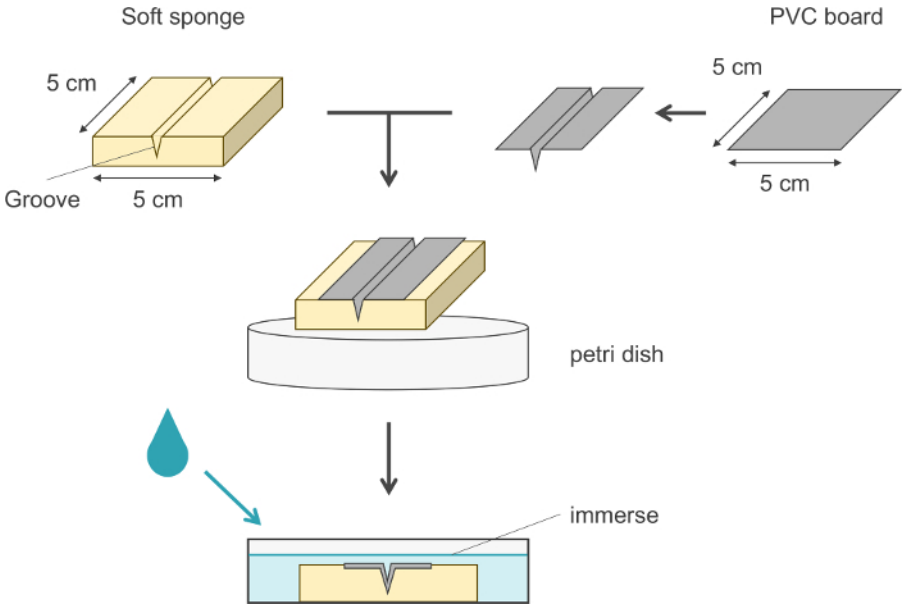

Figure 2: A View Illustrating the Surgical Table. Please click here to view a larger version of this figure.

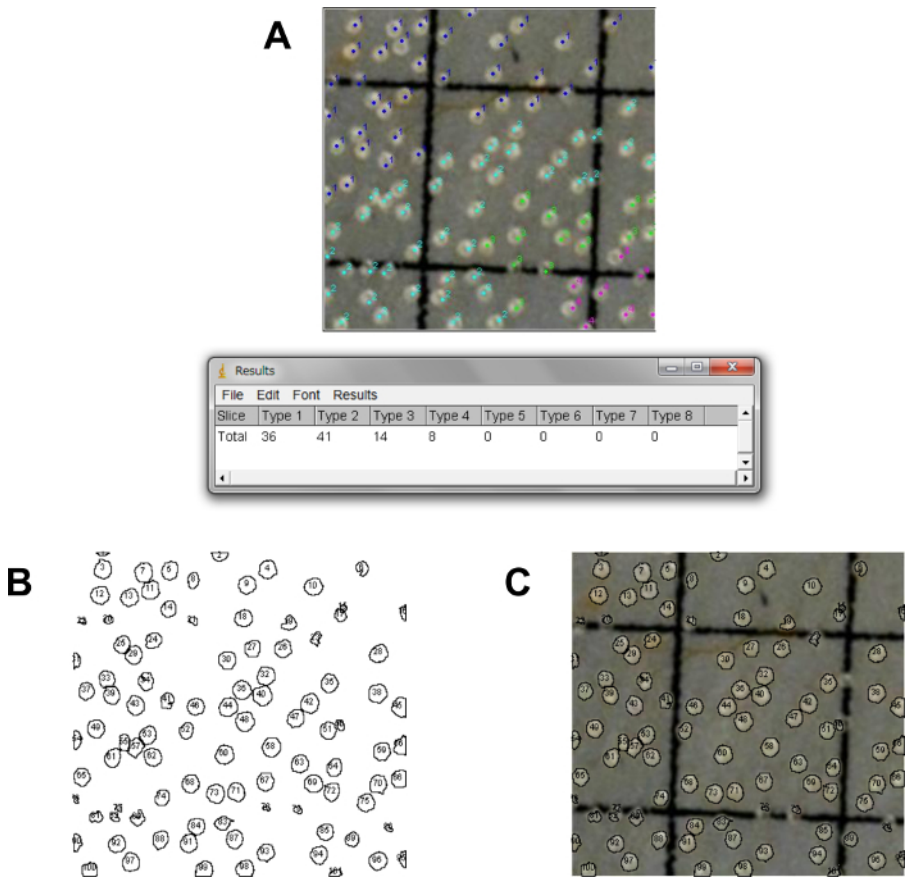

Figure 3: Manual and Automatic Egg Counting using ImageJ. (**A**) Manual counting using Cell Counter plugin. This plugin enables to count eggs grouped by some subdivision. It is an example which eggs were subdivided in four groups and counted. (**B**) Automatic egg counting. (**C**) Image which was merged the automatic egg counting image and the original picture. Please click here to view a larger version of this figure.

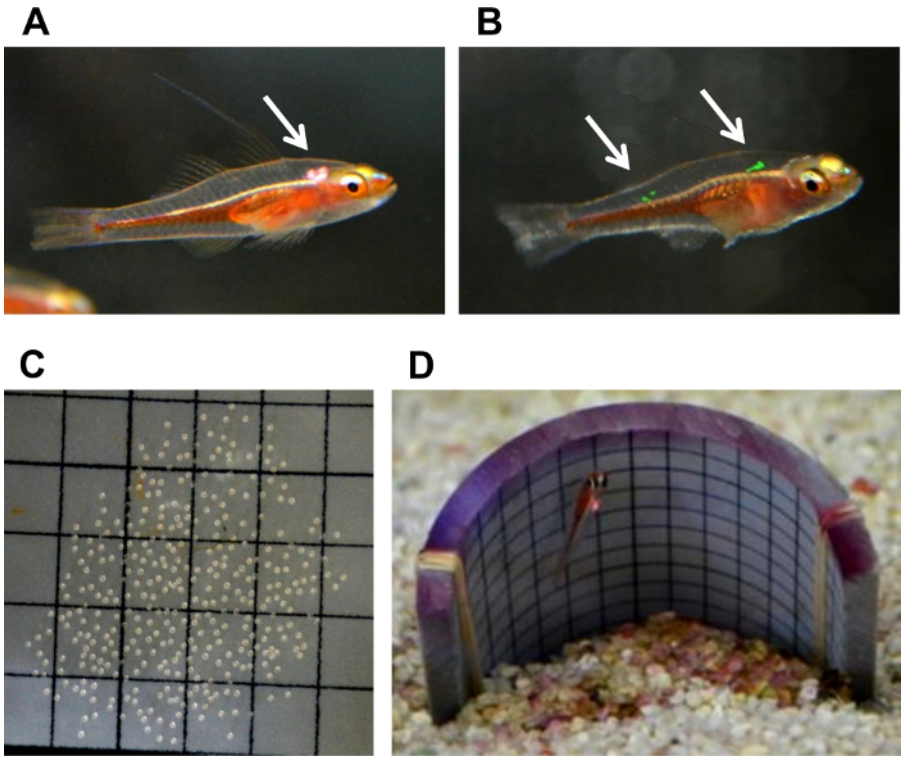

Figure 4: Representative Individual Identification by Injecting *T. marinae* with VIE Tags, and using a Waterproof Sheet for Counting Eggs Deposited on the Artificial Spawning Site. (**A**) Individual No. 1, identified by the pink VIE tag; a *white arrow* indicates the injected VIE tag. (**B**) Individual No. 11, identified by the two green VIE tags; the *white arrows* indicate the injected VIE tags. (**C**) Spawned eggs on a waterproof sheet. (**D**) Paternal care resumed by a male after the sheet with eggs had been removed and photographed, and then placed back into the aquarium. Please click here to view a larger version of this figure.

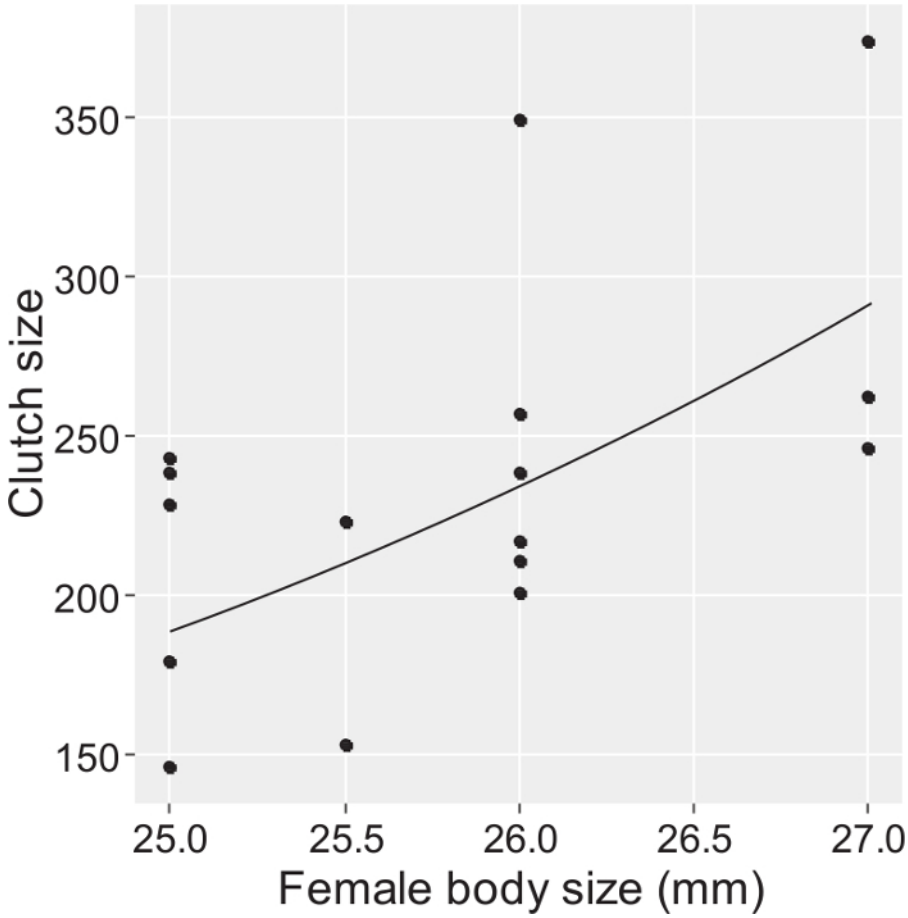

Figure 5. Relationship Between Clutch Size and Female Body Size (Total Length) in *T. marinae*. *Solid curve*, estimated fecundity in each size group of females, obtained with a generalized linear mixed-effects model. These results indicate that female reproductive success increased with body size (Pearson's correlation, $r = 0.56$, $P < 0.05$, $n = 16$). This figure has been modified from Fukuda *et al.*[3] Please click here to view a larger version of this figure.

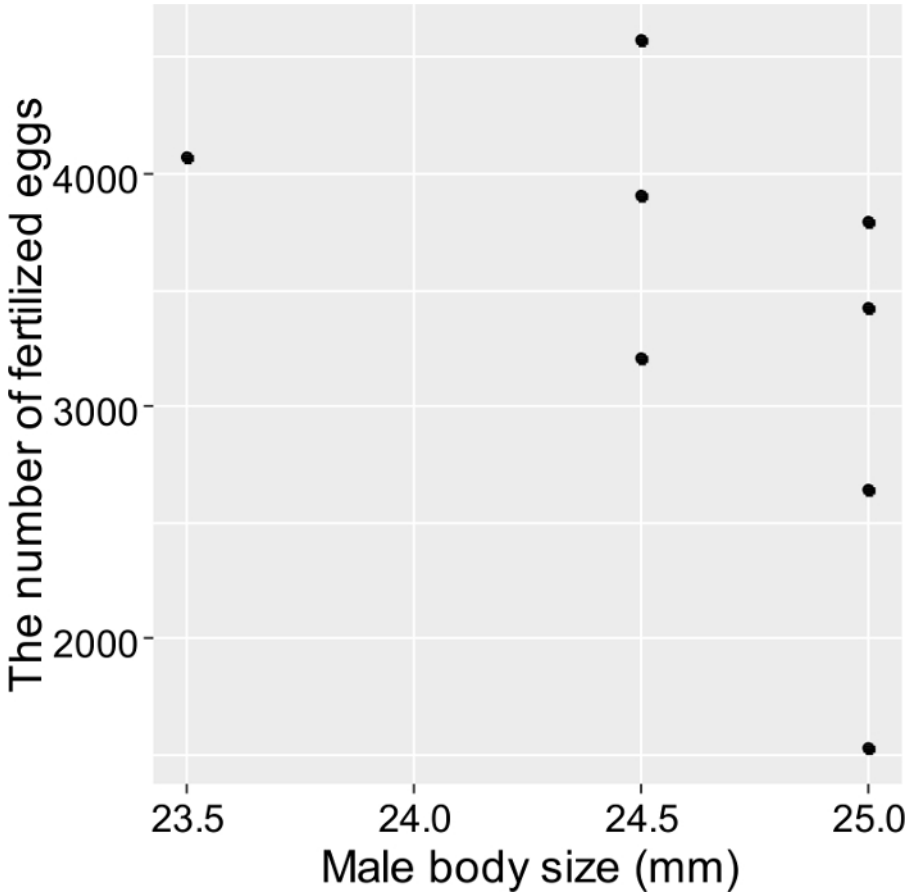

Figure 6. Relationship Between Estimated Mating Success and Male Body Size (total length) in *T. marinae*. Each data item was estimated from the reproduction frequency of males and the estimated fecundity of females. These results indicate that males were reproductively successful irrespective of their body size (Pearson's correlation, r = -0.51, P >0.05, n = 8). This figure has been modified from Fukuda *et al.*[3] Please click here to view a larger version of this figure.

Discussion

The reproductive ecology of numerous fishes has often been revealed through experimental rearing. Especially, sex change[6,8,14], mate choice[15,16] and intraspecific competition[7,17] have been frequent topics of detailed investigations using aquarium-kept fish. Furthermore, some results observed in aquaria have been later confirmed in the field[8,18]. These outcomes support the utility and credibility of rearing experiments with wild-caught fish in aquaria. In addition, manipulation through rearing experiments that simulate situations that may occur naturally but only rarely in wild conditions is worthwhile as a preliminary stage to larger-scale field investigations.

The protocol describes methods suitable for a small-sized substrate spawner that deposits adhesive eggs. Large variations in optimum conditions for aquarium-kept fish can be expected among species, thus warranting adjustments to some points of the protocol. In particular, five points of the protocol should be considered for adjustment after a preliminary assessment of the particular study species: (1) the time spent surfacing the fish, in protocol 1.4; (2) the concentration of the anesthesia liquid and the time spent in the anesthesia just before injecting the VIE tag, in protocols 3.1.3 and 3.1.5, respectively; (3) the needle's insertion depth when injecting the VIE tag into the fish, in protocol 3.1.8; (4) the size and form of the PVC pipe used as an artificial nest site, in protocol 3.2.1; and (5) using three egg counting methods properly, in protocol 4.6: manual count (accurate but takes time and effort), automatic count and area-density ratio estimation (efficient but rough). When there are few eggs, or when the counting grouped by some subdivision is need (such as dead or alive, developmental stage-based classification and so on), the manual counting method is recommended. When there is a large number of eggs, and the ImageJ can distinguish each egg individually, the automatic counting method may be suitable. The area-density ratio estimation is effective when there are many, and densely eggs and the ImageJ can't distinguish each egg individually.

Many species of fish may not perfectly maintain their buoyancy after being brought to the surface. However, careful surfacing according to this protocol may allow most fish to recover within one day. If fish are found floating upside down in the collection bag just after surfacing, wait to determine whether the fish is dead at least one day before removing it. If fish die soon after being brought to the surface or if they need more than one day to recover, surface more slowly or extend each time interval during surfacing in the course of subsequent collecting efforts.

In addition to VIE tags, other methods exist for identifying individual fish: colored external plastic streamers, nylon anchor tags, fin clipping, and passive integrated transponder (PIT) tags, *etc*. However, especially when collecting small fish, some of these techniques may increase mortality, hinder growth, or cannot be visible *in situ*.[10] Moreover, as most external tags protrude from the fish body, a tag may restrict some behaviors of species that inhabit burrows, narrow crevices or dense seaweed beds. In contrast, many studies of small fishes found that VIE tagging had

no major negative effect on mortality and growth[10,11]. VIE tags may also negligibly effect fish behavior since the subcutaneous tag does not protrude, however small the fish is, making it an especially suitable identification method for behavioral observations of small-sized species[10]. According to some previous studies, the acrylic paints also can be used in the same way as the VIE tags[19,20].

Artificial spawning nest is generally used for the investigation of reproduction of fishes which spawn the demersal adhesive eggs. Previous studies used artificial nests, which are made from different kinds of materials, such as the terracotta roof tile[21], the ceramic tile[22], the shell[23], PVC box[24], *etc*. These artificial spawning nests may be useful for many substrate spawners. These studies suggest that availability of the artificial nest for fish, such as the shape and/or size, is more important than what it is made from. As the PVC pipe is the material that is easy to obtain and process, this paper used the PVC pipe as the spawning nest.

The limits of ecological information gained through captive-rearing observations should be well appreciated. Unsurprisingly, rearing in aquaria, in comparison with a species' natural environment, restricts various ecological conditions of the aquatic habitat (*e.g.*, physical and chemical features of the water, food ecology, opportunities for intra- and inter-specific interactions, habitat extent, and population density). It may lead individuals to exhibit particular behaviors that differ from their natural ones. Therefore, field investigations should complement rearing observations so as to provide the best background for inferring adaptive evolution of fish reproductive behaviors.

Disclosures

The authors have nothing to disclose.

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References

1. Helfman, G., Collette, B. B., Facey, D. E., Bowen, B. W. *The Diversity of Fishes: Biology, Evolution, and Ecology.* Wiley-Blackwell, Oxford (2009).
2. Davies, N. B., Krebs, J. R., West, S. A. *An Introduction to Behavioural Ecology.*, 4th edit. Wiley-Blackwell, Oxford (2012).
3. Fukuda, K., Manabe, H., Sakurai, M., Dewa, S., Shinomiya, A., Sunobe, T. Monogamous mating system and sexuality in the gobiid fish, *Trimma marinae* (Actinopterygii: Gobiidae). *J Ethol.* **35** (1), 121-130 (2017).
4. Manabe, H., Matsuoka, M., Goto, K., Dewa, S., Shinomiya, A., Sakurai, M., Sunobe, T. Bi-directional sex change in the gobiid fish *Trimma* sp.: does size-advantage exist? *Behaviour.* **145** (1), 99-113 (2008).
5. Sakurai, M., Nakakoji, S., Manabe, H., Dewa, S., Shinomiya, A., Sunobe, T. Bi-directional sex change and gonad structure in the gobiid fish *Trimma yanagitai*. *Ichthyol Res.* **56** (1), 82-86 (2009).
6. Sunobe, T., Nakazono, A. Sex change in both directions by alteration of social dominance in *Trimma okinawae* (Pisces: Gobiidae). *Ethology.* **94** (4), 339-345 (1993).
7. Wong, M. Y., Munday, P. L., Buston, P. M., Jones, G. P. Monogamy when there is potential for polygyny: tests of multiple hypotheses in a group-living fish. *Behav Ecol.* **19** (2), 353-361 (2008).
8. Kuwamura, T., Suzuki, S., Tanaka, N., Ouchi, E., Karino, K., Nakashima, Y. Sex change of primary males in a diandric labrid *Halichoeres trimaculatus*: coexistence of protandry and protogyny within a species. *J Fish Biol.* **70** (6), 1898-1906 (2007).
9. Winterbottom, R. Two new species of the *Trimma tevegae* species group from the Western Pacific (Percomorpha: Gobiidae). *Aqua J Ichthyol Aquat Biol.* **10**, 29-38 (2005).
10. Frederick, J. L. Evaluation of fluorescent elastomer injection as a method for marking small fish. *Bull Mar Sci.* **61** (2), 399-408 (1997).
11. Olsen, E. M., Vøllestad, L. A. An evaluation of visible implant elastomer for marking age-0 brown trout. *N Am J Fish Manag.* **21** (4), 967-970 (2001).
12. Leblanc, C. A., Noakes, D. L. Visible implant elastomer (VIE) tags for marking small rainbow trout. *N Am J Fish Manag.* **32** (4), 716-719 (2012).
13. Kinkel, M. D., Eames, S. C., Philipson, L. H., Prince, V. E. Intraperitoneal injection into adult zebrafish. *J Vis Exp.* **42**, e2126 (2010).
14. Reavis, R. H., Grober, M. S. An integrative approach to sex change: social, behavioural and neurochemical changes in *Lythrypnus dalli* (Pisces). *Acta Ethol.* **2** (1), 51-60 (1999).
15. Milinski, M., Bakker, T. C. Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature.* **344**, 330-333 (1990).
16. Basolo, A. L. Phylogenetic evidence for the role of a pre-existing bias in sexual selection. *Proc R Soc Lond B Biol Sci.* **259**, 307-311 (1995).
17. Milinski, M. Optimal forging: the influence of intraspecific competition on diet selection. *Behav Ecol Sociobiol.* **11** (2), 109-115 (1982).
18. Manabe, H., Ishimura, M., Shinomiya, A., Sunobe, T. Field evidence for bidirectional sex change in the polygynous gobiid fish *Trimma okinawae*. *J Fish Biol.* **70** (2), 600-609 (2007).
19. Stammler, K. L., Corkum, L. D. Assessment of fish size on shelter choice and intraspecific interactions by round gobies *Neogobius melanostomus*. *Environ Biol Fishes.* **73** (2), 117-123 (2005).
20. Matsumoto, Y., Tawa, A., Takegaki, T. Female mate choice in a paternal brooding blenny: the process and benefits of mating with males tending young eggs. *Ethology.* **117** (3), 227-235 (2011).
21. McCormick, M. I. Behaviorally induced maternal stress in a fish influences progeny quality by a hormonal mechanism. *Ecology.* **79** (6), 1873-1883 (1998).
22. Knaepkens, G., Bruyndoncx, L., Coeck, J., Eens, M. Spawning habitat enhancement in the European bullhead (*Cottus gobio*), an endangered freshwater fish in degraded lowland rivers. *Biodivers Conserv.* **13** (13), 2443-2452 (2004).

23. Shiogaki, M., Dotsu, Y. The life history of the gobiid fish, *Zonogobius boreus. Bulletin of the Faculty of Fisheries, Nagasaki University.* **37**, 1-8 (1974).
24. Meunier, B., Yavno, S., Ahmed, S., Corkum, L. D. First documentation of spawning and nest guarding in the laboratory by the invasive fish, the round goby (*Neogobius melanostomus*). *J Great Lakes Res.* **35** (4), 608-612 (2009).

