**Morphology of a Novel Reproductive Strategy in the pelvic-brooding ricefish *Oryzias eversi* Herder et al. 2012 (Adrianichthyidae, Teleostei)**

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**ABSTRACT**

We examined reproductive features of a pelvic brooder, the oviparous teleost, *Oryzias eversi*. Eggs possess two types of filaments that form on the surface of the cholion during oogenesis: long attachment filaments at a restricted area around the vegetal pole, and sparse, short filaments around the animal pole (micropyle). Females spawned eggs through the genital pore when mating with the male. After spawning, females carry a cluster of fertilized eggs suspended from the genital pore by a bundle of long attachment filaments. Fertilized eggs were protected by the pair of long pelvic fins and held in an abdominal concavity posterior to those fins until hatching, about 12 days post-oviposition. During the period that females carried the fertilized egg cluster, the growth of oocytes slowed, and ovulation of a new batch of eggs did not occur. However, if the current brood was accidentally lost, the next oviposition occurred sooner than normally expected. Thus, reproductive cycles in the female of *O. eversi* appear to depend upon the presence of hanging embryos.

**Key words**: *Oryzias eversi*, attachment filament, pelvic brooder, ovulation, oviposition cycle

**INTRODUCTION**

The genus *Oryzias* (Family, Adrianichthyidae) comprises approximately 40 species and ranges broadly throughout fresh and brackish waters of Central, North and Southeast Asia and the Indo-Malay-Philippines Archipelago as far east as Timor (Parenti, 2008; Fricke et al., 2025). The oviparous mode of reproduction in *Oryzias* is categorized as lecithotrophic. Most of these oviparous fishes exhibit photo-periodically synchronized ovulation. Under natural lighting conditions during the breeding season, mature females of *Oryzias latipes* lay eggs in the early morning every day. If enough males to stimulate mating behavior are present, females lay eggs almost immediately after ovulation (Robinson and Rugh, 1943; Egami, 1954). According to Egami (1954), the time of oviposition may be modified when fish are artificially exposed to a sufficient photoperiod.

The eggs of fishes in four families in the order Beloniformes - Adrianichthyidae, as well as Scomberesocidae, Hemiramphidae and Exocoetidae - are spherical and have filamentous appendages on the cholion (egg envelope, *zona radiata* or *zona pellucida*) (Rosen and Parenti, 1981; Collette et al., 1984). Two types of filaments are present on the surface of the egg envelops of *Oryzias* species: long attachment filaments (AFs) restricted to the vegetal pole area (VPA) and short filaments on the rest of the cholion. The AFs on spawned eggs often become entangled with and attached to objects floating in the water column (Yamamoto, 1975). In *O. latipes,* females frequently carry spawned eggs until the subsequent spawning. Most *Oryzias* species, including *O. latipes*, are called “transfer brooders” (Spanke et al., 2021) because females deposit spawned eggs on aquatic plants or solid surfaces. Yet, in some oviparous *Oryzias* and *Adrianichthys* species, the AFs appear not only to keep the embryos attached to the female until they hatch but also to regulate the reproductive cycle. These species are called “pelvic brooders” (Kottelat, 1990).

The evolution of viviparity established specialized endocrinological maternal-fetal relationships (Wourms, 1981). In the diversity of reproductive strategies, it is not surprising to find that fishes exhibit nearly all the reproductive modes found among other vertebrates. Even in oviparous fishes, the reproductive cycle is regulated by the presence of embryos, as reported for mouth-brooding cichlids (Specker and Kishida, 2000). Pelvic brooders have been documented in at least five species of ricefishes, family, Adrianichthyidae, all from the Indonesian island of Sulawesi: *Oryzias sarasinorum* (Popta, 1905; Iwamatsu and Sato, 2004; Iwamatsu et al., 2007), *O. eversi* (e.g., Herder et al., 2012; Mokodongan and Yamahira, 2015; Schüller et al., 2022), *O. kalimpaaensis* (e.g., Gani et al., 2022), *Oryzias bonneorum* (e.g., Möhring et al., 2025), and *A. oophorus* (Kottelat, 1990). Females of these species carry a cluster of developing embryos in an abdominal concavity posterior to the urogenital papilla (protuberance) until the embryos hatch. The fertilized eggs are suspended via AFs held together by a plug that forms in the oviduct (Schüller et al., 2022). In these pelvic brooders, females never ovulate while they carry fertilized eggs and developing embryos (Iwamatsu et al., 2007, 2008). The reproductive behavior of pelvic brooders differs from that of transfer brooders which spawn photo-periodically every day. The present study focused on the morphological and reproductive features of the pelvic brooder *O. eversi*. Schüller et al. (2022) reported precise histological investigations of this phenomenon including form of the plug. To further understand the reproductive cycle, we measured the sizes of follicles (oocytes) and post-ovulatory follicles in a single ovary. Our observations show that *O. eversi* females that carry embryos attached to the abdomen, oocytes develop during the vitellogenic phase of oogenesis and, therefore, that oocyte maturation and ovulation do not occur during pelvic brooding.

**MATERIALS AND METHODS**

**Animals**

In the present study, 3 females (43.7 ± 3.5 mm in total length, TL) and 5 males (43.9 ± 2.6 mm TL) of *Oryzias eversi* kept in the World’s Medaka Aquarium, Higashiyama Zoo, Nagoya-city, Japan, were examined. Adult females were kept under reproductive conditions (13 hours light, 6:00-19:00, 27 ℃) and fed on live larvae of *Propsilocerus akamusi* and a standard dry diet (Otohime, Marubeni Nisshin Feed Co., Ltd, Tokyo, Japan). Females carrying a cluster of spawned eggs were deeply anesthetized in a mixture of water-saturated phenylurethane (7 parts) and ethanol (3 parts) and examined anatomically under a binocular dissecting microscope (SZX12, Olympus, Tokyo, Japan). The anesthetized females were then euthanized by pithing with the sharp tip of ophthalmic scissors. After the abdominal cavity was opened, the ovary was isolated and quickly fixed in cold (4 ℃) 0.05% glutaraldehyde in regular saline (NaCl 6.5 mg/ml, KCl 0.4 mg/ml, CaCl2·2H2O 0.15 mg/ml, adjusted to pH 7.4 with 0.1N NaHCO3) for medaka oocytes (Iwamatsu, 2018). This study was conducted following the Guidelines for the use of fishes in research by Use of Fishes in Research Committee (2014).

**Observations of oocytes, eggs and pelvic girdles**

Females carrying a cluster of fertilized eggs by a bundle of attachment filaments were deeply anesthetized as described above, pithed, and laparotomized. The ovaries were removed in saline with fine forceps and a scalpel. Pelvic girdles, with pelvic fins attached, were stained with 0.001% alizarin red S, followed by 0.5 % KOH. These samples were cleared in 50% glycerol for observation. The stages of oogenesis reported here are in accordance with stages in *O. latipes* (see Iwamatsu et al., 1988), although the size of oocytes varies.

**Histological examination and 3D reconstruction of female genital organ**

The fish were anaesthetized and fixed in Davidson’s fixative (22% of a 37% solution of formaldehyde, 33% ethanol, 11.5% glacial acetic acid, and 33.5% distilled H2O) at room temperature and stored until further processed. The fixed samples were dehydrated with ethanol, embedded in paraffin (Parabett, Muto Pure Chemicals Co., Ltd., Tokyo, Japan), and the entire fish was sectioned sagittal (5 μm) using a sliding microtome (REM-700, Yamato Koki Industrial Co., Ltd., Saitama, Japan). The sections were stained with haematoxylin and eosin (HE) for light microscopy observation. A three-dimensional (3D) image of the genital organs was reconstructed from the coronal sections using ReconstructTM 1.1.10 (Fiala, 2005) and converted to a PDF file using PDF3D ReportGen (VTS software K.K., Tokyo, Japan).

**RESULTS**

**Traits of sexual dimorphism**

Adult males of *O. eversi* have 6 to 9 black spots on their lateral body surface (Fig. 1A), as previously described by Herder et al. (2012). Males have an elongate anal fin, a dorsal fin with long fin-rays, and an indistinct, or no urogenital papilla (UGP). In contrast, adult females are characterized by having smaller anal and dorsal fins with relatively short fin-rays, along with long pelvic fins and a well-developed single-lobed UGP (Figs. 1B, 2A, B and C). The elongated pelvic fins enable females to hold and protect a cluster of spawned eggs in their inconspicuous belly concavity (Figs. 1A, B and Fig. 2A). The distal tips of the long pelvic fins extend posterior to the anterior part of the anal fin (Figs. 1B and 2A). The female has a well-developed pelvic girdle (Fig. 2D), which functions in moving the pelvic fins to protect the hanging embryos. The length of the anal fin in males (mean 29.2 ±0.42 % of standard length: SL, n = 8) was significantly greater than that of females (mean 18.2±5.2 % of SL, n = 3, Fig. 3A). In sexually mature males of *O. eversi*, the tip of the anal fin has long fin rays without a fin fold (membrane) in the distal region. The fin rays in the posterior portion of the anal fin lack papillae, and the notch that separates the last fin ray from the anterior fin rays is absent in the anal and dorsal fins in the males, in contrast to the males of *O. latipes* (Fig. 1A). The number of dorsal fin rays is 9 to 12. The length of the dorsal fin with 11 fin rays was significantly greater in males (mean 24.2 ± 1.8 % of SL, n = 7) than in females (mean 16.4 ± 1.0 % of SL, n = 3) (Fig. 3A). *O. eversi* males use their dorsal and anal fins to grasp gently but firmly the caudal fin of female at mating as do male *O. latipes*. We also observed that the pectoral fin in males was longer than in females (Fig. 3A).

In contrast, the pelvic fins of *O. eversi* females were significantly longer (16.1 ± 2.5 % of SL, n = 3) than those of males (11.5 ± 0.41% of SL, Fig. 3A). The pelvic fins of females have 6 fin rays, and the total number of ray nodes (connections of segments) was 111.0 ± 10.7 (range 93 – 130, n = 3) and significantly greater than that (85.0 ± 4.9, n =7) in males (Fig. 3B). The total number of ray ramification in the pelvic fin of females was 6.3 ± 0.88 (range 5 - 8, n = 3) and greater than that in males (5.3 ± 0.64, range 3 - 8, n =7)) (Fig. 3C).

**Female genital organ and ovary**

On the ventral surface of the the adult brooding female, the anus opens at the anterior region of the UGP, which has intricate folds on its surface (Fig. 2B). Three-dimensional reconstruction of serial sections of the tissue revealed that the digestive tube is largely curved, and the anus opens at the anterior end the UGP (Fig. 2C). However, the orifices of the oviduct and urinary duct open at the posterior end of the UGP (Fig. 2A, 2C and 2D). The ovarian cavity connects to the short oviduct, which has a sphincter with a thick wall (Fig. 2D).

Shortly after oviposition, the ovary (2.9 - 3.4 mm in width, 3.4 mm in length) of mature females consists of the stromal compartment and the dorsal ovarian cavity, which is surrounded by the ovarian wall with intricate folds as described by Schüller et al. (2022). The dorsal surface of the ovary connects to the median surface via the mesovarium, which attaches to the dorsal peritoneum; the width of the ovary on the left side of the mesovarium is less than on the right side. In the stromal compartment, oocytes in various stages (I – VIII, Figs. 2D and 5) of oogenesis within all the various-sized developing follicles and the post-ovulatory follicle (POF) are observed. These follicles and POFs connect with the follicular stalks, which are located on the opposite side of the follicle attaching to the ovarian wall (oe) and anchor the respective follicles to the abdominal reticular connective tissue (rt) (Fig. 4B). In a female (45 mm TL) at 5 days post - oviposition, the tangled mass of AF contained two types of interstitial (or epithelial) cells within the inner wall of the ovarian cavity (Schüller et al., 2022), and the AF mass hardened into a compact plug (1.0 - 1.5 mm in diameter) of the genital pore (Fig. 2D).

**Reproductive cycle**

After the spawning, females carry a cluster of fertilized eggs hanging in their abdomen by long AFs and continue to carry the developing embryos for 10-13 days (mode = 12 days) during the period from spawning to hatching in aquarium (Fig. 5). During pelvic brooding, the female often shakes its body by moving the pelvic fins as if it senses the presence of hanging embryos and is thus stirring the water surrounding the embryos.

**Morphological features of growing oocytes and eggs**

The volume of ooplasm gradually increases as oocytes grow. All oocytes are anchored to the abdominal ovarian rete by thread-like follicular stalks (Figs. 4A and 4B). A line connecting the follicular stalks and the center of the follicle area attaching to the ovarian wall is the "follicle axis" as described in detail for *O. latipes* (see Iwamatsu et al., 2020). The large spherical egg nucleus (germinal vesicle, about 48 µm in diameter) contains many chromatin threads and variously sized nucleoli in the peripheral region of the nucleus (perinucleolar stage: stage II) (Fig. 5B). On the surface of the cholion of oocytes greater than 135 µm in diameter, the primordia of attachment filaments (AFs) are first detectable as minute wart-like bumps in the presumptive vegetal pole area (VPA) of the oocyte (Fig. 5C). The inner layers of the cholion are not yet formed in the young oocyte of this developmental stage. In the cytoplasm of such oocytes, there is a single yolk nucleus (Balbiani body), which differs from the ubiquitously formed some nuages in the juxtanuclear cytoplasm (Hamaguchi, 1987) (Fig. 5B, C). In oocytes of ca. 250 µm in diameter, oil droplets appeared in the cortical cytoplasm adjacent to the nucleus (oil droplet stage VI) (Fig. 5D). When oocytes grow larger than 300 µm in diameter, about one-third of the oocyte surface is covered by elongated AFs that are wound in the opposite direction of oocyte rotation. In the oocyte at this stage, the tips of all AFs on the surface of the cholion uniformly bend at right angles to the animal-vegetal (A-V) axis (the hypothetical line connecting the micropyle to the center of the area covered by AFs). As vitellogenesis proceeded, small yolk globules fused with each other to form a single spherical mass (yolk sphere), and the diameter of the spherical mass gradually increased with the growth of oocytes (Fig. 6). The layer of granulosa cells surrounding the VPA became thicker as compared to that covering the animal hemisphere.

**Changes in AFs and the oocyte composition in an ovary following oviposition**

On the day after oviposition, females (35 mm total length: TL) were carrying a batch of fertilized eggs at the embryonic shield stage 17 (1,497.4 ± 23.4 µm in diameter, n = 63). The distal parts of AFs formed a tangled mass and adhered to the inner surface of the ovarian cavity. The micropyle (Fig. 7A) is detectable on the cholion directly opposite the VPA (Fig. 7B). On the surface of each egg, the number of small villi (mean 95.4 ± 0.4 µm in length, range 10 - 450 µm, n = 40) was 16.0 ± 0.7 (n = 20). These short villi are restricted to a small area (ca. 450 µm x 180 µm in diameter) around the micropyle (Figs. 7A and C). The total number of oocytes was 2,440 in an ovary (longitudinal 6.14 mm, lateral 2.93 mm). The largest population of stage I oocytes occupied 60.3% in an ovary, and nearly full-grown oocytes of vitellogenic stage IX (larger than 800 µm in diameter) (range 846 - 1,026 µm) were only 1.2% (Fig. 8). The mean size of post-ovulatory follicles (n = 15) was 741.7 ± 47.6 µm in length, 214.2 ± 26.8 µm in width and 67.1 ± 18.6 µm in thickness.

In a female (45 mm TL) at 5 days post - oviposition, the total number of oocytes in its ovary was counted to be 1,820. In this female, the ovary exhibited a conspicuous decrease of the smallest oocytes in stage I (33.4%), whereas the number of oocytes in stage II had increased (31.5%, Fig. 8) and the number of nearly full-grown oocytes (0.1%) remained unchanged. The oocytes slowly progressed from stage I into stage II - V; most of them remained at the early stages of oogenesis, with a diameter less than 251 - 400 µm, as was seen in the ovary on the day after spawning, and full-grown oocytes larger than 800 μm in diameter were rare. Thus, the range of sizes of oocytes in such an ovary exhibited the previtellogenesis-vitellogenesis pattern (stage I – VIII of oocytes) and lacked fully grown and matured oocytes 5 days post - oviposition, like that on the day after oviposition. The mean size of POFs decreased to 486.0 ± 41.2 µm in length and 261.0 ± 44.4 µm in breadth in the ovary at this time point.

The cluster of developing embryos (developmental stage 34 on 5 days post - oviposition) (mean diameter 1,562 ± 13.2 µm; range 1,525 - 1,630 µm, n = 14) was kept near the anterior end of the anal fin by long AFs (13.0 ± 0.6 in number, n = 19) as in the female shown in Fig. 1. The long AFs were present on the restricted area (about 216 x 189 µm) of the surface of the cholion in VPA (Figs. 7B - E). Just after hatching, AFs remained attached to the thin residue (outermost layer) of the cholion that had not been hydrolyzed by hatching enzymes.

**DISCUSSION**

The egg can be a critical factor in studying the evolutionary diversity of reproductive strategies. Although the morphology of developing oocytes in *O. eversi* is like that of *O. latipes* oocytes (Iwamatsu et al., 1988; Iwamatsu and Nakashima, 1996), the two species differ significantly in the role of fertilized eggs in controlling the development and oviposition of eggs. Females of *O. latipes* spawn eggs at dawn (shortly before the onset of the light phase) and carry a cluster of spawned eggs in the belly held by AFs for 10 hours or longer after spawning. If the spawned eggs are not removed by the action of the female in swimming and contacting the roots of water plants and so on, they are shed when the AFs are pushed out of the oviduct by subsequently spawned eggs. In such females, the proximal portions of the AFs do not transform into a complex structure that plugs the oviduct; as a result, the AFs are expelled, and the embryos float away from the female at the time of next oviposition.

 In a group of the pelvic-brooders, *O. eversi* Herder et al., 2012 (Hilgers et al., 2022; Schüller et al., 2022; the present study), *O. kalimpaaensis* Gani et al., 2022, and *O. sarasinorum* (see Iwamatsu et al., 2008), - a loosely tangled mass of the tips of AFs remains in the ovarian cavity, adhering to the inner wall of the ovarian cavity via mucus secreted just after oviposition. Hilgers et al. (2022) and Schüller et al. (2022) hypothesized that inflammation caused by the retention of AFs in the ovarian cavity triggers formation of the oviduct plug. The epithelial (interstitial) cells lining the inner wall of the oviduct appear to migrate among the tangled tips of AFs during inflammation (Hilgers et al., 2022; Schüller et al., 2022) and narrow the oviduct. Consequently, female *O. eversi* carry fertilized eggs via the AFs until the embryos hatch.

In oviparous *O. eversi*, ovulation does not occur during a period of pelvic brooding for the 12 days that females carry developing embryos, apparently in a way that mimicks pregnancy in viviparous animals. We have no evidence that the plug–structure in this species functions like a placenta during pregnancy, a close relationship between ovarian activity and the presence of the plug–structure (or developing embryos in the belly) can be inferred from the experimental observation that the artificial removal of embryos (AFs) from the belly shortens the intervals of the reproductive cycle in *O. sarasinorum* (see Iwamatsu et al., 2007). This uncertainty includes whether and when an endocrine signal secreted by the cells of the plug affects the development of oocytes. Whatever the processes involved, the presence of the plug, including the maternal tissue in it, appears to inhibit or retard oogenesis and the next oviposition. This situation resembles the relationship between the retardation of oogenesis and the parturitions in viviparous fish species. The inhibitory effect of embryos on the development of oocytes has long been reported in viviparous fishes (Turner, 1937). For example, in the poeciliid *Gambusia affinis*, young oocytes grow slowly until the next gestation and reach the maturation stage within about 5 days after parturition (Koya et al., 2000). This inhibitory effect of ovarian gestation on oocyte maturation seems to resemble the effect of cross-talk with the plug-like structure in *O. sarasinorum* and *O. eversi*, as estimated by the size of oocytes in an ovary while the plug is present in the oviduct. Thus, the oviductal plug may be specialized to accomplish the two functions: to carry the cluster of embryos and to regulate the maturation of oocytes.

 In *O. latipes* females that are artificially inseminated in the ovarian cavity by introducing a sperm suspension shortly after ovulation, fertilized eggs with a hardened cholion fail to be squeezed out of the ovarian cavity through the genital pore (Iwamatsu et al., 2005, 2022). As a result, the fertilized eggs begin to develop within the ovarian cavity. Such “pregnant” females continue to ovulate every morning, despite the presence of developing embryos in the ovarian cavity, and ovulated eggs accumulate in the ovarian lumen at subsequent ovulations every morning.

Our study will provide biologists and aquarists with unexpected but interesting knowledge that AFs form and elongate on the cholion at the vegetal pole region (VPA) with a thick follicular layer of young oocytes, and wind around the vegetal hemisphere in fully grown oocytes as oocytes grow. The tuft of long AFs on the cholion and Balbiani body (yolk nucleus) in the cytoplasm (Fig. 5D) mark the vegetal pole of the egg and is a diagnostic trait of *Oryzias* species belonging to the family Adrianichthyidae, although its number is different among them (Iwamatsu, 2018; Iwamatsu et al., 1988, 2020). As we demonstrate, the eggs of *O. eversi* also have a left- or a right-handed rotation on the animal-vegetal (A-V) axis confirmed by the tips of AFs bending in a uniform direction. In these eggs, the A-V axis is recognized as a line that connects the vegetal pole (AFs) with the opposite animal pole (micropyle), and it is implied that the egg polarity of *O. eversi* oocytes is determined in the same manner as that of *O. latipes* oocytes, which also rotate on the A-V axis of the oocyte during oogenesis (Iwamatsu, 1994; Iwamatsu et al., 2020). Other common features among the eggs of species of the genus *Oryzias* include the short villi (non-attaching filaments) distributed on the surface of the egg enevelope except for the VPA (see Iwamatsu, 2018). Eggs of *O. eversi* have a few very short villi in only the restricted area around the micropyle. The eggs of another pelvic brooder, *O. sarasinorum*, are also morphologically similar with respect to the scarceness of villi, a trait that might be the result of phylogenetic loss. The eggs of the pelvic brooder *Adrianichthys oophorus* have no short villi on the cholion (Kottelat, 1990). Pelvic brooders appear to display a similarity in developmental patterns of the villi on the cholion..

Several pelvic brooders spawn very large eggs compared to transfer brooders, e.g., *O. eversi* (egg diameter is 1.4 mm in Herder et al., 2012; 1.46 ± 0.02 mm in the present data), *O. kalimpaaensis* (2.19 ± 0.10 mm in Gani et al., 2022), *O. sarasinorum* (2.09 ± 0.01 mm in Iwamatsu et al., 2008) and *Adrianichthys oophorus* (2.0-2.1 mm in Kottelat, 1990). The features of the egg confirm a phylogenetic relationship as reported by Rosen (1964) and Rosen and Parenti (1981). The developmental period of 12 days in *O. evers*i, in which the diameter of the egg is about 1.5 mm (present study), is greater than the 9 days (26 ℃) in the transfer brooding *O. latipes* (1.2 mm in diameter). In *O. sarasinorum* with larger eggs (2.1 mm in diameter), the developmental period is far longer (15 days). Thus, the developmental period of eggs may have a close relation to the egg size (yolk volume). That is, in pelvic brooders, the period that female pelvic brooders carry their fertilized eggs appears to be a function of the size of their eggs.

In common with many transfer brooders in the genus *Oryzias* - *O. curvinotus*, *O. latipes*, *O. luzonensis*, and *O. mekongensis* (see Iwamatsu, 2018) - the male possesses longer anal and dorsal fins than the female. Moreover, numerous small papillary processes on the inter-segments in the posterior region of the long fin rays of the male anal fin are well known (Oka, 1931; Yamamoto and Egami, 1974, cf. Yamamoto, 1975). The presence of the papillary processes in the long fin rays of the anal fin also has been reported in some transfer brooders as a sexual characteristic. This sexual character is absent in males of the pelvic brooders, *O. sarasinorum*, *O. eversi*, *Adrianichthys oophorus* and *A. poptae* (unpublished), and also in other transfer brooders in Sulawesi, Indonesia as well as *O. dancena* in India.

In addition to this trait, sexual dimorphism in the length of pelvic fins — shorter in males than in females - is confirmed commonly in most species of *Oryzias*, including transfer brooders such as *O. celebensis*, *O. javanicus* and *O. latipes* and in pelvic brooders such as *O. evers*i and *O. sarasinorum* (see Iwamatsu, 1986). In pelvic brooding species of *Oryzias* — including *O. sarasinorum* (see Iwamatsu and Sato, 2004; Iwamatsu et al., 2007), *O. eversi* (see Schüller et al., 2022) and *O. kalimpaaensis* Gani et al., 2022, - as well as *Adrianichthys poptae* (Weber and Beaufort, 1922) and *A. oophorus* (Kottelat, 1990), females use the distal tips of their elongated pelvic fins, which meet beyond the origin of the anal fin.

Several traits of female pelvic brooders enable this common reproductive strategy, especially the long pelvic fins that protect the cluster of embryos from predators during the long developmental period. Also, in pelvic brooders such as *O. eversi* (the present study) and *O. sarasinorum* (see Iwamatsu et al., 2008), the pelvic girdle of females, which structurally supports the long pelvic fins, are larger than those of males. Further study of other sexually dimorphic traits, e.g., pleural ribs (Spanke et al., 2021), will be necessary to understand the diversity in evolution of ricefishes.

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**FIGURE LEGENDS**

Fig. 1. Living *Oryzias eversi* and a cluster of developing embryos hanging from a female's abdomen by a tuft of attachment filaments.

(A) A sexual pair of *O. eversi* exhibiting traits of sexual dimorphism. Scale bar = 10 mm.

(B) The pelvic region during pelvic brooding. Developing embryos (about 6 days after spawning) hanging at the posterior end of urogenital papilla (ugp) by long attachment filaments (arrowheads). Pelvic fins (pf) underhanging embryos. af, anal fin. Scale bar = 1 mm

Fig. 2. Morphology of abdominal region and germinal organs of *O. eversi* female.

(A) Lateral view of abdominal region: long pelvic fins (pf) are overhanging beyond the anterior base of anal fin (af). Broken end of attachment filaments (α) is seen at the end of a single urogenital papilla (ugp). af, anal fin; pf, pelvic fins. Scale bar = 1 mm.

(B) A single-lobed urogenital papilla of a female is viewed from directly below. Arrowhead indicates the anus (an) in the anterior region of a urogenital papilla (ugp), which has a complex pattern of color and ridges. Scale bar = 100 µm.

(C) A 3D image of female genital organs was constructed from serial histological sections. green, oocytes; brown, digestive tract; magenta, plug-like structure; blue, body wall; red, spawned eggs. an, anus; ugp, urogenital papilla. PDF file with 3D content is supplemented online. Scale bars = 1 mm.

(D) Longitudinal section of abdominal region with germinal organs of *O. eversi* female brooding embryos. Hematoxylin-eosin stained. dt, digestive tract; gc, genital cavity; gv, germinal vesicle; ov, ovary; ow, ovarian wall; sh, sphincter-like structure of the oviduct; sy, spherical yolk (yolk mass) ; ub, urinary bladder. Scale bar = 500 µm.

(E, F) In a female (E), a pair of left (L) and right (R) elements of pelvic girdles (PG) with pelvic fins (PF) exhibits more specialized parts facing each other in comparison with male ones (F). Scale bar = 1 mm.

Fig. 3 Sexual dimorphism of fins in *O. eversi*.

(A) Ratios of fin length / standard length of fish (SL, standard length) (%) in anal fin and dorsal fin were larger in males than females, while those in pelvic fin was larger in females than males.

(B) Number of ray nodes (connection of segments) in anal fins and dorsal fins were greater in males, while those in pelvic fin were greater in females than males.

(C) Total number of ray ramification in dorsal fin was larger in males, while those in anal fin and pelvic fin were almost equal in females and males.

Fig. 4 Oocytes and post-ovulatory follicle in the ovary of *O. eversi*.

(A, B) In ovary, follicles with growing oocytes (A) and post-ovulatory follicles (pof, B) are anchored by follicular stalks (fs) to the reticular connective tissue (rt). Follicular stalks (fs) attach the opposite side of ovarian epithelium (oe). ap, animal pole; vp, vegetal pole. Scale bar = 200 µm.

Fig. 5. Frequency distribution of the day from spawning to hatching and histological sections of oocytes of *O. eversi*.

(A) Females continue to carry developing embryos for 10-13 days (mode = 12 days) from spawning until the embryos hatch.

(B, C) Histological section (hematoxylin-eosin stained) of stage II (B) and stage III (C) oocyte. There is a single yolk nucleus (Balbiani body, arrows in B) in the cytoplasm of the oocytes in the stage II. The primordia of attachment filaments (AFs) are first detectable as minute wart-like bumps in the presumptive vegetal pole area (VPA) of the oocyte (arrowheads in C). AP, animal pole; gv, germinal vesicle ; VP, vegital pole. Scale bar = 50 µm.

(D) In an ovary of a female that was carrying developing embryos, oocytes in various stages (I - VII) of oogenesis were observed within all the various-sized developing follicles in which elongated AFs in a thick follicular layer on the chorion in the vegetal pole area and yolk nucleus (Balbiani body, arrows) in the cytoplasm were detectable (arrowheads). Scale bar = 100 µm.

Fig. 6 Changes in a spherical yolk mass within vitellogenic oocytes of *O. eversi*.

Small yolk globules fused with each other to form a single spherical mass (yolk sphere) in oocytes at an early yolk formation stage or later as vitellogenesis proceeded and the diameter of the spherical mass increased in proportion to the diameter of oocytes.

Fig. 7 Developing embryos being carried by female.

(A) Long attachment filaments are absent, and only small villi are present in the animal pole area of the chorion of embryos being carried by female on the day after oviposition. m, micropyle: v, villi; o, oil droplet. Scale bar = 100 µm.

(B) Attachment filaments (α) are present in only a restricted area near the vegetal pole (VPA) of the chorion of fertilized eggs of *O. eversi*. Scale bar = 100 µm.

(C) The animal-vegetal (A-V) axis connects the micropyle (arrowhead) at the center of a region where the chorion is thickened (animal pole area) to the center of the area where the attachment filaments (α) are formed (vegetal pole area). Small arrows indicate the small villi in the animal pole area of the embryos (stage 17) on the day after oviposition. Scale bar = 250 µm.

(D) The fertilized eggs developed into embryos in the developmental stage 34 at 5 days after oviposition onto the belly of spawned female. The micropyle (arrowhead) and attachment filaments (α) can still be seen. cd, Cuvier duct; ha, atrium of heart; hv, ventricle of heart; sv, sinus venosus; vc, median yolk vein. Scale bar = 250 µm.

(E) Embryos were artificially removed from the female just before hatching. The basal portion of the shortened attachment filaments (α) are localized to the vegetal pole area of the chorion (c). Scale bar = 100 µm.

Fig. 8 Diameters of oocytes in an ovary of *O. eversi* females carrying developing embryos.

(A) In the ovary of the female carrying fertilized eggs 1 day after oviposition, the most abundant oocytes were at stage I and the most abundant; oocytes at stage VI (251 – 400 µm in diameter) were the second most abundant, suggesting that after oviposition, oogenesis is arrested at these two stages.

(B) In the ovary of the female carrying fertilized eggs 5 days after oviposition, oocytes at stage I decreased and oocytes at stages II, III, IV and V increased, suggesting that oogenesis was proceeding but slowly.