**A Novel Strategy of Reproduction in *Oryzias eversi* (Adrianichthyidae, Teleostei)**

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**ABSTRACT**

We examined reproductive features in a pelvic brooder, oviparous teleost, *Oryzias eversi*. Eggs possess two types of filaments that form on the surface of the chorion 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 by mating with the male. After spawning, females carry an egg cluster suspended from the genital pore by a bundle of long attachment filaments. Fertilized eggs were protected by a pair of long pelvic fins in an abdominal concavity posterior to the pelvic fins until hatching about 12 days post-oviposition. During the period that females carried the 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 it normally would have. Thus, reproductive cycles in the female of *O. eversi* appear to depend entirely upon the presence of hanging embryos.

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

**INTRODUCTION**

The genus *Oryzias* (Family, Adrianichthyidae) comprises at least 24 species, which ranges broadly throughout fresh and brackish waters of Central, North and Southeast Asia and the Indro-Malay-Philippines Archipelago as far east as Timor (Parenti, 2008). The oviparous mode of reproduction in *Oryzias* is categorized as lecithotrophic. Most of these oviparous fishes exhibit photo-periodically synchronized ovulation. Under the 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 the mating behavior is present, females lay eggs almost immediately after ovulation (Robinson and Rugh, 1943; Egami, 1954). According to Egami (1954) and Yoshioka (1964), the time of oviposition can be modified whenever if fish are artificially exposed to a sufficient photoperiod.

The eggs of fish in four families in the order Beloniformes - Adrianichthyidae, as well as Scomberesocidae, Hemiramphidae and Exocoetidae - are spherical and have filamentous appendages on the chorion (egg envelope, *zona radiata*) (Rosen and Parenti, 1981; Collette et al., 1984). In the eggs of *Oryzias* species, there are two types of filaments: long attachment filaments (AFs) restricted to the vegetal pole area (VPA), and short filaments on the rest of the chorion. The AFs on spawned eggs 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 solids. However, in some of oviparous *Oryzias* and *Xenopoecilus* species, the AFs appear not only keep the embryos attached to the female until they hatch but also regulate the reproductive cycle. These species are generally called “pelvic brooders” (Kottelat, 1990).

Fishes are the most diversified vertebrates with respect to their morphology and ecology (Wourms, 1981). Particularly, the ovulation of viviparity establishes the specialized endocrinological maternal-fetal relationships. In the diversity of reproductive strategies, it is not surprising to find that fishes exhibit nearly all the reproductive modes found in the other vertebrates. Even in the oviparous fishes, reproductive cycle is regulated by embryos as reported in the mouth-brooders (Specker and Kishida, 2000). So far, pelvic brooders have also been reported in two monophyletic genera, *Adrianichthys* and *Oryzias* (Parenti, 2008); *Oryzias sarasinorum* (Pota, 1905; Iwamatsu and Sato, 2004; Iwamatsu et al., 2007), *O. eversi* (Herder et al., 2012; Mokodongan and Yamahira, 2015; Schüller et al., 2022) and *O. kalimpaaensis* (Gani et al., 2022) as well as *Adrianichthys poptae* (Weber and Beautfort, 1922), and *A. oophorus* (Kottelat, 1990). These females carry a cluster of developing embryos in the posterior region of the genital papilla (protuberance) in the abdominal concavity by AFs on the chorion until the embryos hatch. In these pelvic brooders, females never ovulate while they carry fertilized eggs and developing embryos (Iwamatsu et al., 2007, 2008). This reproductive event of the pelvic brooders completely differs from that of the transfer brooders in which spawning takes place photo-periodically every day. The present study focused on the morphological and reproductive features of the pelvic brooder *O. eversi*. Schüller et al. (2022) have reported excellent histological investigations of this phenomenon. In order to understand the reproductive cycle, we measured the sizes of follicles (oocytes) and post-ovulatory follicles in a single ovary. Our observations show that in *O. eversi* females carrying embryos attached to the abdomen, oocytes development proceeded slowly during the vitellogenic phase of oogenesis and therefore, that oocyte maturation and ovulation do not take place during the 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 using a 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, aCl2·2H2O 0.15 mg/ml, adjuzted to pH 7.4 with 0.1N NaHCO3) for medaka oocytes (Iwamatsu, 2018). This study was conducted following the Guidance for the Use of Fishes, in Research (the American fisheries Society).

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

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

**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 longitudinal-sectioned (5 μm) throughout the whole body 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. Three-dimensional (3D) image of the genital organs were reconstructed from the cross sections using ReconstructTM 1.1.10 (Fiala, 2005) and converted to PDF file using PDF3D ReportGen (VTS software K.K., Tokyo, Japan).

**RESULTS**

**Traits of sexual dimorphism**

Adult males of *O. eversi* exhibit 6-9 blackish spots on their lateral body surface (Fig. 1A), as previously described by Herder et al. (2012). They have the elongated anal fin and dorsal fin with long fin-rays, and have small urogenital papilla (UGP) which is absent in some males. In contrast, adult females are characterized by having less developed anal fin and dorsal fin with 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 help the 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 are hanging over the anterior part of the anal fin (Figs. 1B and 2A). Anatomical observations also showed that the female has a pair of the well-developed pelvic girdles (Fig. 2D), which play a role in moving the pelvic fins to protect the hanging embryos. The length of anal fin of males (mean 30.0±2.1% of standard length :SL, n = 8) were significantly longer than those of females (mean 18.2±5.2 % of SL, n = 3, Fig. 3A). In sexually mature males of *O. eversi*, the tip of anal fins have long fin rays without a fin fold (membrane) at the distal region. The fin rays in the posterior region of the anal fin lack the papilla processes and the notch which 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 fin rays of dorsal fin is 9 - 12 and the length of the dorsal fin with 11 fin rays was significantly longer in the males (mean 24.7 ± 1.9 % 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 for grasping gently but firmly the tail of female at mating in the same way as *O. latipes* males do.

In contrast, the pelvic fins of *O. eversi* females were significantly longer (16.1 ± 2.5 % of SL, n = 3) than those of males (11.8 ± 0.27 % of SL, Fig. 3A). The female pelvic fins 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 larger than that (88.0 ± 7.4, n =7)) in males (Fig. 3B). The total number of ray ramification in female pelvic fin was 6.3 ± 0.9 (range 5 - 8, n = 3) and larger than that (5.3 ± 0.6, range 3 - 8, n =7)) in males (Fig. 3C).

**Female genital organ and ovary**

In the belly of the adult brooding female, an anus opens at the anterior region of the UGP with the intricate folds on their surface (Fig. 2B). 3D reconstruction of serial sections of the tissue revealed that the digestive tube is largely bent and the anus opens toward the front on the UGP (Fig. 2C). On the other hand, the orifices of 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 lumen being surrounded by the markedly modified inner wall of the oviduct (Fig. 2D).

The ovary (2.9 - 3.4 mm in width, 3.4 mm in length) of mature females shortly after oviposition consists of the stromal compartment and the dorsal ovarian cavity which is surrounded by ovarian wall with the 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, and 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 anchored respective follicles to the abdominal reticular connective tissue (rt) (Figs 4B). In a female (45 mm TL) at 5 days post - oviposition, the tangled mass of AF contains the two types of interstitial (or epithelial) cells within the inner wall of the ovarian cavity (Schüller et al., 2022), and the AF mass became hardened as a compact plug (1.0 - 1.5 mm in diameter) of the genital pore as in the female shown in Fig. 2D.

**Reproductive cycle**

After the egg spawning, females carry a cluster of spawned eggs hanging in their abdomen by long Afs and continue to carry the developing embryos for 10-13 days (mode = 12 days) in the period from spawning to hatching in aquarium (Fig. 5). During pelvic brooding, female often shakes its body by moving the pelvic fins as if recognizing the presence of hanging embryos and 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 B) and a line connecting the follicular stalks and the center of the follicle area attaching to the ovarian wall is so-called follicle axis as described in detail in *O. latipes* (Iwamatsu et al., 2020). The large spherical egg nucleus (germinal vesicle, about 48 µm in diameter) contains many chromatin–threads and various sized nucleoli, distributed in the peripheral region of the nucleus (perinucleolar stage: stage II) (Fig. 5B). On the surface of the chorion (egg envelope) of oocytes larger than 135 µm in diameter, the primordia of attachment filaments (AFs) are detectable as minute wart-like bumps for the first time in the presumptive vegetal pole area (VPA) of the oocyte (Fig. 5C). The inner layers of the chorion 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 a third part of the oocyte surface are covered by winding of elongated Afs and the direction of oocyte rotation is ascertainable by state of FAs of the oocyte. In the oocyte at this stage, the tips of all AFs on the surface of the chorion 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 of 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 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 chorion directly the opposite of VPA (Fig. 7B). On the chorion 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 present only in a restricted 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 (n = 4), and nearly full-grown oocytes of vitellogenetic 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%), while 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 very slowly progressed from stage I into stage II - V; these oocytes remained at the early stages of oogenesis with diameter of 251 - 400 µm or less, 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 oocytes composition in an ovary exhibited the previtellogenesis-vitellogenesis pattern (stage I – VIII of oocytes) without full grown and matured oocytes 5 days post - oviposition, similar to 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 chorion surface in VPA (Figs. 7B - E). Just after hatching, AFs remained attached to the thin residue (outermost layer) of the chorion that had not been hydrolyzed by hatching enzymes. The rotatory direction of oocytes was indicated by left- or right-handed bending of the AFs on the slightly thin chorion (Fig. 7F).

**DISCUSSION**

The egg can be a critical factor in studying evolutionary diversity of reproductive strategies. Although the morphology of developing oocytes in *O. eversi* is quite similar to that of *O. latipes* oocytes (Iwamatsu et al., 1988; Iwamatsu and Nakashima, 1996), the two species differ significantly in the role of fertilizing 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 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 roots of water plants and so on. They are shed when the AFs are pushed out of oviduct by subsequently spawned eggs. In such females, 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 at the latest.

 Unlike the Afs in such species, in other species - *O. eversi* (Herder et al., 2012; Hilgers et al., 2022; Shüller et al., 2022; the present study), *O. kalimpaaensis* (Gani et al., 2022), and *O. sarasinorum* (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. (2012) and Shüller 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; Shüller et al., 2022) and narrow the short oviduct. Consequently, female *O. eversi* carry fertilized eggs with such AFs until the embryos hatch.

In oviparous *O. eversi*, ovulation dose not occurs during the 12 days that females carry developing embryos in their belly, apparently in a way mimicking the pregnant period in viviparous animals. At present, although we have no evidences as to whether the function of the plug–structure in this fish may correspond to the placenta during the pregnancy, a close relationship between the 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* (Iwamatsu et al., 2007). This uncertainty includes 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 been reported in viviparous fishes (Turner, 1937). For example, in *Gambusia affinis*, young oocytes grow slowly grow up to 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: carrying the cluster of embryos and regulating 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 chorion (egg envelope) fail to squeezed out of the ovarian cavity through the genital pore (Iwamatsu et al., 2022). As a result, the fertilized eggs begin to develop within the ovarian cavity. However, 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 each ovulation at every morning.

Our study will provide the aquarists and biologists with much unexpected but interesting knowledges that AFs form and elongate on the chorion at the vegetal pole region (VPA) of young oocytes, winding around the vegetal hemisphere in fully grown oocytes as oocytes grow. The tuft of long AFs on the chorion can check visually (indicate) the vegetal pole of the egg and is a phylogenetic trait of *Oryzias* species belonging to the family Oryziatidae, although its number is different among them (Iwamatsu, 2018; Iwamatsu et al., 1988, 2020). As shown in the present study, the eggs of *O. eversi* also indicate a left- or a right-handed rotation on the animal-vegetal (A-V) axis that is ascertainable with a visual evidence that the tips of AFs indicate the bending to 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 in *O. latipes* oocytes which rotate on the A-V axis of the oocyte during oogenesis (Iwamatsu, 1994; Iwamatsu et al., 2020). Further, the other common features among the eggs of the genus *Oryzias* fishes is the short villi (non-attaching filaments) distributed on the surface of the chorion except for the VPA (see Iwamatsu, 2018). Eggs of *O. eversi* possess a few very short villi in only the restricted area around the micropyle. As seen in another pelvic brooder *O. sarasinorum*, eggs of this pelvic brooder also are morphologically similar with respect to the scantiness of villi which might be the result of phylogenetic loss. The egg of the pelvic brooder, *Xenopoecilus oophorus*, have no short villi on the chorion (Kottelat, 1990). Pelvic brooders appear to display a similarity in developmental patterns of the villi on the chorion of eggs.

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 *Xenopoecilus oophorus* (2.0-2.1 mm in Kottelat, 1990). The features of the egg seem to support a phylogenetic relationship reported by Rosen (1964) and Rosen and Parenti (1981). The developmental period of 12 days in *O. eves*i of which the egg size was about 1. 5 mm as observed at the present time is longer than that of 9 days (26 ℃) in the transfer brooder *O. latipes* with small size (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 the pelvic brooders, the period that female pelvic brooders carry their fertilized eggs in their bellies appears to be dependent on the size of their eggs.

In common with many transfer brooders in the genus *Oryzias* - *O. curvinotus*, *O. latipes*, *O. luzonensis*, *O. mekongensis* (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 long fin rays of the anal fin also has been reported in some transfer brooders as a sexual characteristic. To contrary, such a sexual character is absent in males of the pelvic brooders, *O. sarasinorum*, *O. eversai*, *Xenopoecilus oophorus* and *X. poptae* (unpublished), and also in many other transfer brooders in Sulawesi, Indonesia as well as *O. melastigma* in India. Therefore, the papillary processes in the anal fin of males are not always a significant sexual characteristics in males. In addition to this trait, a sexual dimorphism that the length of pelvic fins is shorter in male in comparison to that of the female is commonly confirmed in most of *Oryzias* species, such transfer brooders as *O. celebensis*, *O. javanicus* and *O. latipes*, and in the pelvic brooders, *O. evers*i and *O. sarasimorum* (Iwamatsu, 1986). In the pelvic brooders of *Oryzias* species including *O. sarasinorum* (Iwamatsu and Sato, 2004; Iwamatsu et al., 2007), *O. eversi* (Shüller et al., 2022) and *O. kalimpaaensis* (Gani et al., 2022) as well as *Xenopoecilus poptae* (Weber and Beautfort, 1922) and *X. oophorus* (Kottelat, 1990), females can use their elongated pelvic fins of which distal tips meet beyond the anal fin origin.

The female traits of pelvic brooders are likely beneficial and gainful in the context of the common reproductive strategy. Especially, the long pelvic fins of these pelvic brooder females protect a cluster of embryos from egg predators for a long developmental period. Therefore, in pelvic brooders such as *O. eversi* (the present data) and *O. sarasinorum* (Iwamatsu et al., 2008) pelvic girdles of females functionally supporting the long pelvic fins develop greater than those of males. Furthermore, detailed observations with the utmost care and attention on sexually dimorphic traits such as pleural ribs (Spanke et al., 2021) will be required for understanding the diversity in evolution of medaka fish. The present results will provide aquarists as well as biologist with much unexpected but interesting knowledges.

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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 (AFs).

(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) are 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 = 0.1 mm.

(C) A 3D image of female genital organs was constructed from a 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.

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

(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) Total numbers of ray node (connection of segments) in anal fin and dorsal fin were larger in males, while those in pelvic fin was larger in females than males.

(C) Total numbers of ray ramification in dorsal fin were larger in males, while those in anal fin and pelvic fin were almost equal between 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) after 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, arrow in B) in the cytoplasm of the oocytes in these oogenesis stages. The primordia of attachment filaments (AFs) are detectable as minute wart-like bumps for the first time in the presumptive vegetal pole area (VPA) of the oocyte (arrowheads in C). Scale bar = 50 µm.

(D) In a 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 of which elongated AFs were detectable (arrowheads). Scale bar = 100 µm.

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

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

Fig. 7 Developing embryos being carried by female.

(A) Long attachment filaments (Afs) 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 only present in the restricted vegetal pole area (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 where the chorion is thicker (animal pole area) and 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 to embryos in the developmental stage 34 at 5 days after oviposition in the belly of spawned female. A micropyle (arrowhead) and attachment filaments (α) can be still detectable. 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 parts of the shortened attachment filaments (α) are localized to the vegetal pole area of the chorion (c). Scale bar = 100 µm.

(F, G) When the trimmed chorion of the embryos artificially removed from the egg just before hatching were observed from above, bases of the attachment filaments (α) shows a right- or left-handed spiral pattern (a right-handed arrow in F and a left-handed arrow in G), indicating the direction of the oocyte rotation during oogenesis in the ovary. Scale bar = 100 µm.

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

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

(B) In the ovary of female carrying fertilized eggs 5 days after oviposition, oocytes at stage I decreased and oocytes at stage II increased, suggesting that the progression of early oogenesis slowed.