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How to stay attached – Formation of the ricefish plug and changes of internal reproductive structures in the pelvic brooding ricefish, *Oryzias eversi* Herder, Hadiaty & Nolte, 2012 (Beloniformes: Adrianichthyidae)

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Data availability statement

The data that support the findings of this study are deposited in the morphology collection of the Museum Koenig in Bonn (Ichthyology collection numbers: Supplementary table 1) and are available upon request.

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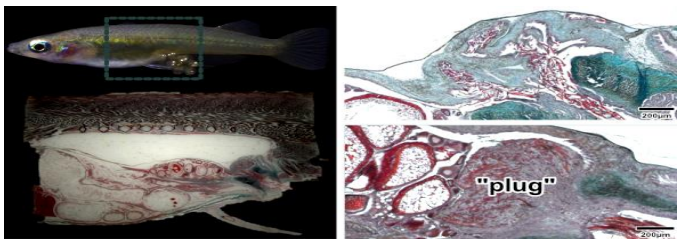
Abstract

Teleost fishes show an enormous diversity of parental care, ranging from no care to viviparity with maternal provisioning of embryos. External brooders carry their

developing eggs attached to their bodies. This requires the formation of novel morphological structures to support attachment. The pelvic brooding ricefish *Oryzias eversi* evolved such a structure, called the "plug". The plug anchors attaching filaments from the fertilized eggs inside the female reproductive system, allowing the female to carry the embryos until hatching. Using histological sections and µ-CT scanning, we show that the plug is formed by several types of interstitial cells, blood capillaries, and collagen fibrils that encapsulate the end of the attaching filaments in the anterior part of the gonoduct. Even 15 days after loss of the protruding attaching filaments, the plug remains. In addition, the developed plug contains multinucleated giant cells that are derived from fusing macrophages. We thus hypothesize that the ricefish plug, which is vital for egg attachment in *O. eversi*, evolved due to an inflammatory reaction. We assume that it forms similar to a foreign body granuloma, as a reaction to irritation or injury of the gonoduct epithelium by the attaching filaments. Our study further corroborates that pelvic brooding entails a complex set of adaptations to prolonged egg-carrying in the female reproductive system. During brooding, for instance, ovulation in the ovary is suppressed and the anterior part of the gonoduct is characterized by an intricate, recessed folding.

Graphical Abstract



In the pelvic brooding ricefish *Oryzias eversi* a unique transient structure, called the plug, forms in the anterior part of the gonoduct after spawning, anchoring the fertilized eggs to the female. Histological sections indicate that the formation of the

plug may be similar to a foreign body reaction, an inflammatory response. Changes of the ovary, the gonoduct or the papilla during brooding likely facilitate pelvic brooding.

Keywords: Egg-attachment, inflammation, foreign body reaction, multinucleated giant cell, histology

Research Highlights: In *O. eversi* an egg-attaching tissue called the plug that forms in the gonoduct involves an inflammatory response. Additional changes in the female reproductive system are hypothesized to be adaptations to pelvic brooding.

1. INTRODUCTION

Various animal species enhance their reproductive success by providing parental care (Gross, 2005; Kölliker, Smiseth, & Royle, 2012). Although teleost fishes show an impressive spectrum of parental care, the majority displays no caring behavior towards their progeny and in most cases of parental care, males guard deposited, fertilized eggs (Gross & Sargent, 1985; Sargent & Gross, 1986). Unstable environmental factors including fluctuating water levels, oxygen content, temperature, or predation pressure may select against stationary guarding especially in freshwater habitats (Baylis, 1981). In specific forms of parental care called "bearing", fertilized eggs are carried, allowing the brooding parent to move freely and escape rough habitat conditions (Balon, 1975, 1981).

Bearing may be divided into internal (viviparity) and external (brooding), depending on whether the developing eggs are carried inside or outside the female reproductive system (Balon, 1975, 1981). In external brooding species, egg attachment is often linked to the evolution of specific morphological structures such as the brood pouch of male pipefishes and seahorses (Syngnathidae; Whittington & Friesen, 2020), the cotylephores of female ghost pipefishes (Solenostomidae; Wetzel & Wourms, 1995), the hook of the nurseryfish (*Kurtus gulliveri*; Berra & Humphrey, 2002), or the filaments of the male skin brooding anglerfish (*Antennarius caudimaculatus*; Pietsch & Grobecker, 1980). Unlike in other viviparous vertebrates, the embryos of viviparous teleosts develop in the ovary and not the female gonoduct (sometimes also referred to as oviduct, even though Müllerian ducts do not develop) (Campuzano-Caballero & Uribe, 2014, 2017; Santamaría-Martín, Plaul, Campuzano-Caballero, Uribe, & Barbeito, 2021; Turner, 1947; Uribe, Cruz, Alarcón, Campuzano-Caballero, & Bárcenas, 2019). A unique brooding strategy, in which an egg-attachment structure develops inside the female gonoduct, evolved in some pelvic brooding ricefishes (Adrianichthyidae; Hilgers et al., 2022; Iwamatsu, Kobayashi, Sato, & Yamashita, 2008).

Ricefishes are a small group of freshwater fishes distributed from East to South and Southeast Asia (Hilgers & Schwarzer, 2019; Parenti, 2008). Female ricefishes carry the spawned, fertilized eggs attached to their body by long attaching filaments that protrude from the genital pore (Figure 1 A, B). The attaching filaments develop from the external zona pellucida (or chorion) of the eggs during oogenesis (Hart, Pietri, & Donovan, 1984; Iwamatsu et al., 2008; Iwamatsu, Ohta, Oshima, & Sakai, 1988; Laale, 1980). Most species of ricefishes are so-called transfer-brooders, as they deposit their eggs on plants or other substrates shortly after spawning (Balon, 1975).

In contrast, a derived brooding strategy called "pelvic brooding" evolved in some ricefishes endemic to freshwaters in Sulawesi, Indonesia (Kottelat, 1990; Mokodongan & Yamahira, 2015). Unlike in the transfer brooding species, the developing eggs of pelvic brooders stay attached to the female until the embryos hatch. Pelvic brooding has been described in three species (*Oryzias eversi*, *O. sarasinorum* and *Adrianichthys oophorus*), from two distantly related lineages (Herder, Hadiaty, & Nolte, 2012; Hilgers & Schwarzer, 2019; Kottelat, 1990; Parenti, 2008). In *O. eversi* and *O. sarasinorum*, the so-called plug, a unique, transient structure forms around the end of the attaching filaments after spawning and anchors the eggs to the female (Figure 1C) (Hilgers et al., 2022; Iwamatsu et al., 2008).

Knowledge of formation and degeneration of the ricefish plug is so far restricted to a single study, conducted on *O. sarasinorum* (see Iwamatsu et al., 2008). In that species, the attaching filaments, mucus, collagen fibrils, cells that were identified as epithelial (interstitial) cells and blood vessels form a compact plug after spawning. Degeneration of the slightly shrunken plug starts after hatching and females are able to spawn again (Iwamatsu et al., 2007, 2008). Surprisingly, the first histological sections and transcriptomic data of brooding *O. eversi* indicated that the formation of the plug may have been triggered by a modified immune response (Hilgers et al., 2022). Thus, understanding how the plug in *O. eversi* is formed, which cells are involved, and which changes of the female reproductive system are necessary, is crucial.

Pelvic brooding is correlated with several morphological modifications including the formation of a ventral body concavity and elongated pelvic fins (Herder et al., 2012; Iwamatsu et al., 2008; Spanke et al., 2021). Still, changes in the reproductive system are barely identified as most knowledge about the morphology of the reproductive

system of ricefishes is based on the *O. latipes* species complex, the medaka, a famous teleost fish model system (Hilgers & Schwarzer, 2019; Iwamatsu, 2015; Iwamatsu, Oda, Kobayashi, Parenti, & Kobayashi, 2020; Nakamura, Kobayashi, Nishimura, Higashijima, & Tanaka, 2010; Robinson & Rugh, 1943; Suzuki & Shibata, 2004; Yamamoto & Suzuki, 1995).

The aim of the present study is to describe the morphology and formation of the ricefish plug and to document cell types involved in plug formation in the pelvic brooding ricefish *O. eversi*. For this, we used µ-computed tomography (µ-CT) scans and histological sections of seven representative time points of the female reproductive cycle of *O. eversi*. We further described general changes in the reproductive system of female ricefishes during brooding and set our data in context with data from other ricefish species.

2. MATERIAL AND METHODS

2.1 Animal keeping and sampling

Mature, captivity bred *Oryzias eversi* Herder, Hadiaty & Nolte, 2012 were maintained in aquaria (23–27°C, 11.5–12.5h illumination, size: 40cm x 50cm x 30cm or 100cm x 30cm x 45cm) at the Museum Koenig in Bonn, Germany. Ricefish stocks date back to animals caught in Sulawesi, Indonesia in 2012. All specimens and tissues were sampled as permitted by the Landesamt für Natur, Umwelt und Verbraucherschutz (§11 Abs. 1Nr 1b, 8a and 8d TierSchG). To observe the formation of the plug and the changes in the reproductive system, 15 females were individually separated in modified net spawning boxes after spawning and sampled at seven representative points of the reproductive cycle. Three females each were collected at one day after spawning **(T1)**, seven days after spawning **(T2)**, one day after hatching of all eggs **(T3),** and three days after the loss of the protruding attaching filaments **(T4)**.

Additionally, one female was sampled at six days after the loss of the protruding attaching filaments **(T5)**, nine days after the loss of the protruding attaching filaments **(T6),** and fifteen days after the loss of the protruding attaching filaments **(T7)**. All females were euthanized in 0.5 % Tricaine mesylate and fixed in either Bouin's fixative (Bouin–Hollande) or 4%-Paraformaldehyde (PFA). During this process the egg bundle of one female (No. 2) sampled one day after spawning fell off. Supplementary online material, Table 1 provides details of the collection, the fixation, and disposition of each specimen.

2.2 Histology

For histology, sampled specimens of the first four time points (T1–T4) were used. After decalcification in 10%-ethylenediaminetetraacetic acid (EDTA) in the refrigerator for 7 days, each female was trimmed with a razor blade, removing the head in front of the pectoral fins and the caudal region anterior to the anal fin. The remaining bodies were manually dehydrated in an ascending alcohol series (80%, 90%, 96%, 100%), cleared in methylbenzoate and butanol, infiltrated with paraffin wax (Histosec®pastilles (without DMSO)) and finally embedded in paraffin blocks. Subsequently, each block was cut into 5µm thick longitudinal sections with a rotary microtome with a water tub (Leica, HistoCore NANOCUT R). The sections were mounted on glass slides, the paraffin removed, and the sections stained with a trichromatic Masson–Goldner stain (light green). Covered slides were observed and photographed using a ZEISS AxioCam "HRC" coupled to a ZEISS microscope (Axio Imager.Z2m). Some images were stacked using the software Zerene Stacker™ (Version 1.04) and the final figure plates were assembled with the vector graphics editor Affinity Designer (Version 1.5.3.69).

2.3 µ-CT scanning

For µ-CT scanning, the sampled specimens of the last three time points (T5–T7) were used. First, the specimens were stained with 1% phosphotungstic acid (PTA) for two weeks. Then, all specimens were washed and transferred into plastic tubes filled with 70% ethanol. To stabilize the fish during scanning, small pieces of polystyrene were added. The abdomen of the specimens was scanned with a Bruker Skyscan 1173 computer topographer with an energy ranging between 40 to 85kV and 89 to 114µA. Supplementary Table 2 provides details for each scan. The scans were analyzed and visualized with Amira (Thermofisher, Version 6.5.0).

3. RESULTS

3.1 Structure of the reproductive system of female Oryzias eversi

The ovary of the pelvic brooder *O. eversi* is an unpaired, sac-like organ in the posterior part of the body cavity (Figure 2). It consists of the ventral stromal compartment, the germinal epithelium, and the dorsal ovarian cavity, and is surrounded by the ovarian wall. The germinal cradles are located between the epithelial cells of the germinal epithelium and the bordering stromal compartment. The stromal compartment contains various stages of developing oocytes, all connected to the germinal epithelium at one side and via follicular stalks to the abdominal ovarian rete on the other side. Thus, the ovary of *O. eversi* is an asynchronous-type. Smaller oocytes were typically in clusters in the peripheral region of the ovary, whereas larger, vitellogenic oocytes tended to be in the middle. In growing oocytes, oil droplets, yolk vesicles, and filaments on the zona pellucida (egg envelope or chorion) were produced. The gonoduct, connecting the ovarian cavity with the genital opening, consists of an anterior (gonoduct lumen) and a posterior (genital cavity) part (Figure 2). The gonoduct lumen is formed by the continuing ovarian wall and characterized by the absence of the germinal epithelium. Several

mucosal folds and a reduction of the lumen characterize the end of the anterior part (Figure 3A). In contrast, the genital cavity is formed by the invaginated epidermis opening up to the anterior gonoduct lumen. A sphincter-like structure, composed of muscle fibers and connective tissue, supports both parts of the gonoduct (Figure 2). The gonoduct and the genital opening are completely separated from the urinary duct and its opening. The anus is located at the anterior part of the genital papilla. In all individuals the papilla was a pronounced protuberance composed of an outer stratified epithelium and an inner vascularized medulla (Figures 2, 3H).

3.2 Changes in the female reproductive system over the course of brooding

Over the course of the different brooding stages, several changes were observed in the reproductive system of female *O. eversi* (Figure 3). First, it was noticeable that the composition of oocyte stages in the stromal compartment of the ovary varied between specimens, especially between specimens of different representative time points (Figure 3A, B). One day after spawning, the ovary was still filled with huge, yolk-rich oocytes (Figure 3A) or large atretic follicles. In contrast, after the loss of the attaching filaments, in one specimen, no vitellogenic and mature oocytes were present at all (Figure 3B). Besides that, one major change occurred in the structure of the wall forming the gonoduct lumen. During brooding the wall was characterized by an intricate, recessed folding (Figures 3C, 4A, 5A), while after hatching of the embryos it was straight (Figures 3D, 6A, 7A). This obvious change of the wall was complemented by micro-anatomical modifications (Figure 3C, D). The first layer of the ovarian wall was an inner single epithelial layer, while the second and thickest layer of the ovarian wall was the tunica albuginea. The tunica albuginea was composed of loose and dense connective tissue fibers as well as smooth muscle cells and is divided into two sub layers. The muscle cells of the inner sub layer seemed to

run longitudinally, whereas the muscle cells of the outer sub layer seemed to run circularly. The tunica albuginea was traversed by blood vessels. In particular during the brooding stages, the luminal epithelium and the tunica albuginea of the ovarian wall were thickened (Figure 3C). The columnar, ciliated epithelial cells were elongated and longer than wide. In contrast, at the non-brooding time points, a thin tunica albuginea was covered for the most part with a simple, thin layer of cuboidal epithelial cells (Figure 3D). In contrast, the stratified epithelium lining the genital cavity, the posterior part of the gonoduct, appeared to be thinner and more invaginated (Figure 3E) during brooding stages than during non-brooding stages (Figure 3F). Finally, the structure of the outer stratified epithelium of the genital papilla changed noticeably. The epidermis of the papilla of most females of the first two time points displayed deep invaginations (Figure 3G, arrows), whereas the epidermis of the papilla of females of the later time points was straight (Figure 3H).

3.3 Formation of the ricefish plug

Over the course of pelvic brooding, a compact egg-anchoring tissue forms in the reproductive tract of female *O. eversi*. On the first day after spawning (Figure 4) the ends of the attaching filaments were located primarily within the gonoduct lumen between intricate, recessed protuberances of the ovarian wall (Figure 4A). The filaments were loosely entangled within each other and several interstitial cells (Figure 4B) and/or a non-cellular greyish mass, probably mucus (Figure 4C). The interstitial cells were unevenly distributed and had a similar coloration as the epithelial cells of the anterior part of the gonoduct, a round cell nucleus as well as a roundish shape. Some cells were bigger and their cell membrane was clearly visible (Figure 4E), whereas smaller cells were grouped together along the filaments (Figure 4F). In the region of the mucosal folds, the attaching filaments punctured the

gonoduct epithelium and pierced the connective tissue underneath (Figure 4D). A few scattered red blood cells were also observed in the gonoduct lumen. The exception to this was specimen 2 (in which the egg bundle fell off); here the gonoduct lumen was mostly empty with the exceptions of a few scattered cells (Figure 3A).

Seven days after spawning (Figure 5), the ends of the attaching filaments and several interstitial cells were still entangled between the protuberances of the ovarian wall in the anterior part of the gonoduct. The developing plug tissue was denser as the number of interstitial cells increased (Figure 5A). Most interstitial cells had the same appearance as one day after spawning. Some of these cells seemed to start to fuse together as indicated by the presence of multiple cell nuclei (Figure 5B, arrow). Also, several thinner and elongated interstitial cells appear to surround the plug tissue along the outermost filaments (Figure 5C). The plug tissue was still mostly separated from the gonoduct. Blood capillaries entered and traversed the plug in the region of the mucosal folds (Figure 5D). The capillaries appeared to develop from the surrounding sphincter-like structure presumably at injured sites of the gonoduct epithelium. The number of interstitial cells, mucus and blood capillaries varied greatly among the three specimens.

One day after the hatching of the fertilized eggs (Figure 6), the entangled mass of attaching filaments, interstitial cells, and blood cells formed a compact plug-like structure in the anterior part of the gonoduct (Figure 6A). At this time point, the ovarian wall was thinner, straight and no longer folded. The attaching filaments were still present, protruded out of the genital opening, but were no longer continuous. In addition, the posterior part of the plug was fused to the inner wall of the anterior part of the gonoduct, the region of the mucosal folds. Blood capillaries traversed not only the fused region of the plug but also the anterior region. Moreover, collagen fibrils

and multinucleated giant cells were observed (Figure 6B, C). The appearance of the multinucleated giant cells varied from containing many unarranged cell nuclei to a few, arranged cell nuclei. In one specimen, the tissue of the plug extended from the gonoduct lumen into the ovarian cavity (lumen between the germinal epithelium and the ovarian wall; Figure 6D) and encapsulated the remains of two ovulated but not spawned oocytes.

3.3 Degeneration of the plug

The first sign of degeneration of the plug was recorded one day after the hatching of the fertilized eggs. Between the filaments passing through the posterior part of the gonoduct, loose interstitial cells were present (Figure 6A). Three days after the loss of the filaments (Figure 7A–C), the plug-like structure was still compact, but was slightly detached from the inner wall of the anterior part of the gonoduct (Figure 7A). At this time point, the plug was more funnel-shaped and accumulations of light red thin, threads could be observed inside (Figure 7B, arrow). Blood vessels, collagen fibrils and multinucleated giant cells were still present. Most interstitial cells forming the developed plug resembled the surrounding epithelial cells in color of stain. The border between the interstitial cells forming the plug and the subsequent epithelial cells of the posterior part of the gonoduct was clearly recognizable (Figure 7C, short arrows). Six, nine and even fifteen days after loss of the protruding attaching filaments, the plug was still present. The plug does not seem to have reduced noticeably in size (Figure 7D). Over time, the connection of the plug with the wall of the anterior part of the gonoduct was decreasing and fifteen days after the loss of the attaching filaments barely recognizable (Figure 7D). A further possible degeneration of the plug or a change of its structure could not be detected by the µ-CT scans.

4. DISCUSSION

Our results confirm that the major difference in the morphology of the female reproductive system between pelvic brooders such as *O. eversi* and *O. sarasinorum* and transfer brooders such as *O. latipes* is the formation of a plug-like structure that anchors the attaching filaments of the fertilized eggs in the gonoduct until the embryos hatch (Iwamatsu et al., 2008). The general morphology of the ovary, the gonoduct, and the genital papilla of the pelvic brooder *O. eversi* is similar to that of the transfer brooder *O. latipes* (see Iwamatsu, 2015; Iwamatsu et al., 2020; Nakamura et al., 2010; Robinson & Rugh, 1943; Suzuki & Shibata, 2004; Yamamoto & Suzuki, 1995). Alterations such as the recessed and intricate folding of the ovarian wall and the suppression of oocyte maturation are hypothesized to be adaptations for pelvic brooding.

4.1 Oocyte maturation in the ovary

Our results indicate a change of composition of oocytes stages in the ovary of *O. eversi,* most likely suppressed maturation during brooding. Likewise in the pelvic brooders *O. sarasinorum* and *A. oophorus* oocyte maturation is suppressed during brooding, as spawning is not possible (Gundo, Rahardjo, Batu, & Hadie, 2016; Gundo, Rahardjo, Lumban Batu, & Hadie, 2013; Iwamatsu et al., 2007). A reduced breeding frequency is a common cost for the brooding parent (Smith & Wootton, 1995). However, the mechanisms of suppression of oocyte maturation in pelvic brooding ricefishes are still unknown. For *O. sarasinorum,* it is assumed that the plug may inhibit oogenesis similar to several viviparous fishes, in which oogenesis is suppressed by the presence of the embryos (Iwamatsu et al., 2008; Turner, 1937). In *O. latipes*, oocyte maturation is under endocrine hormone control. For instance, hypophysectomy and the administration of sex steroids results in a blockage of oocyte maturation (Iwamatsu, 1978; Iwamatsu & Akazawa, 1987). In addition, gonadotropin-

stimulated granulosa cells play a major role in inducing oocyte maturation (Iwamatsu, 1980). Furthermore, analyses of the blood of daily spawning female *O. latipes* revealed diurnal changes of the concentration of 17β-Estradiol, which regulates the expression of a follicle-stimulating hormone (Kayo, Oka, & Kanda, 2020). We assume it is likely that endocrine hormone control also alters oocyte maturation in pelvic brooding species and that the presence of the plug and/or the developing embryos influences the timing of their development, but this needs further investigation.

4.2 Changes in structure of the gonoduct may facilitate pelvic brooding

The gonoduct of female *O. latipes* is tubular, non-glandular, and so far, unique among teleosts, as it consists of an anterior and a posterior part which develop separately (Suzuki & Shibata, 2004). Our data confirm that the posterior part of the gonoduct of female *O. eversi* is not formed by the ovarian wall, but, instead, by a stratified epithelium. This might not only protect the connective tissue underneath, but also prevents the plug from forming here. During brooding, the intricate recesses of the anterior part of the gonoduct of *O. eversi* together with the inner columnar epithelial cells may be beneficial to hold onto the filaments until the plug is formed. In *O. latipes*, microanatomical changes of the ovarian wall are only reported for the ovarian cavity and here the main function of the inner epithelial cells is secretion to facilitate the extrusion of the eggs (Takano, 1968; Yamamoto, 1963). We also observed a strong sphincter-like musculature of the gonoduct and mucosal folds in female *O. eversi* which may also help to keep the filaments inside the body during plug formation. Mucosal folds are so far only reported for viviparous species in which they reduce the gonoduct lumen (Campuzano-Caballero & Uribe, 2014; Santamaría-Martín et al., 2021). Likewise, a musculature surrounding the gonoduct seems not to be

present in many teleost species (Uematsu & Hibiya, 1983). Histological data on the gonoduct morphology of further ricefish species will shed light on the evolution of modified gonoduct structures in pelvic brooding.

4.3 The genital papilla of female Oryzias eversi

In *O. eversi*, the genital papilla is a female-positive sex-character and, as in all pelvic brooding ricefish species, single lobed (Herder et al., 2012; Parenti, 2008). Form and size differences between male and female papillae are described for many species of ricefish (Magtoon & Termvidchakorn, 2009; Mandagi, Mokodongan, Tanaka, & Yamahira, 2018; Parenti & Hadiaty, 2010; Parenti, Hadiaty, Lumbantobing, & Herder, 2013), but only for *O. latipes* is it reported that the female papilla grows during the breeding season (Yamamoto & Suzuki, 1995). Our histological sections indicate that in female *O. eversi* the main change over the course of the reproductive cycle relates to the structure of the stratified epithelium, which especially after spawning displayed deep invaginations. We thus assume that the invaginations may facilitate egg-carrying until the plug is formed. There are several examples from other teleost fishes with female oviposition-specific papilla modifications (Castro, Gonzales, & Camacho, 2019; Cole & Parenti, 2021; Martin & Page, 2015). In species of the live-bearing poeciliid *Gambusia*, epidermis folds of the papilla function as a guiding structure for the uptake of sperm bundles (Greven, 2005).

4.4 Is the formation of the ricefish plug in Oryzias eversi *a foreign body reaction?*

The ricefish plug is a transient tissue in the female reproductive tract enabling the female to carry the fertilized eggs until hatching. We investigated how during brooding the attaching filaments become encapsulated and thus anchored in the gonoduct. Tissue-specific transcriptome data of *O. eversi* indicate that this reaction might be a modified immune response (Hilgers et al., 2022). Hence, we hypothesize

that the formation of the plug is based on a foreign body reaction. A foreign body reaction is a chronic inflammatory response to foreign bodies such as splinters, prostheses or dermal fillers, which are too large to be phagocytized and thus become encapsulated (Coleman, King, & Andrade, 1974; Lee & Kim, 2015). Besides the size, shape or chemical components of the foreign body, its movement may also trigger the immune system (Coleman et al., 1974; Veiseh et al., 2015). The attaching filaments, or the remains of the ovulated egg inside the ovarian cavity, are not true "foreign bodies", as they have a maternal origin (Iwamatsu et al., 1988), but they could provoke a similar reaction through mechanical stimulation or injury of the gonoduct wall (Hilgers et al., 2022). If the plug still is, or has been derived from, a foreign body granuloma, the interstitial cells are likely epithelioid cells, derivatives of macrophages (cells of the immune system) forming an epithelioid tissue, which resembles epithelial tissue (Ross & Pawlina, 2011). In teleosts, epithelioid cells also have epithelial cell characteristics, which reinforces their striking resemblance (Noga, Dykstra, & Wright, 1989). There are only a few types of multinucleated giant cells, which differ in appearance, formation, and function (Anderson, 2000; Aterman, Remmele, & Smith, 1988; Brodbeck & Anderson, 2009). Both the foreign body and the Langhans' giant cells are characteristic of a macrophage lineage (Okamoto, Mizuno, & Horio, 2003). Thus, the presence of multinucleated giant cells in the plug of *O. eversi* further supports our foreign body reaction theory. It has already been demonstrated that the formation of multinucleated giant cells and thus the foreign body reaction in teleost fishes is similar to those observed in mammals and that a granuloma includes epithelioid type cells and foreign body giant cells (Manrique, Figueiredo, Belo, Martins, & Moraes, 2017; Secombes, 1985). Specifically, in the pacu, *Piaractus mesopotamicus* (Serrasalmidae) and the zebrafish, *Danio rerio* (Cyprinidae) implanted coverslips induced the formation of foreign body giant cells and Langhans'

giant cells (Belo et al., 2021; Belo et al., 2005; Gurevich, French, Collin, Cross, & Martin, 2020). Hence, our results support the assumption that the ricefish plug may be one of few examples of a stress-induced evolutionary innovation (Hilgers et al., 2022; Wagner, Erkenbrack, & Love, 2019).

4.5 When is the plug lost in Oryzias eversi*?*

In contrast to transfer brooding ricefish species, pelvic brooding females do not spawn every morning. Female *O. sarasinorum* spawn again shortly after hatching of the embryos, presumably after the plug is lost (Iwamatsu et al., 2008). Accordingly, it seems that the plug degenerates within a few days. Surprisingly, our µ-CT scans show that in *O. eversi* the plug is still present even fifteen days after the loss of the (externally visible) filaments. Therefore, we assume that the plug might not only be responsible for anchoring the fertilized eggs during brooding, but also for closing the gonoduct opening and thus preventing an internal infection e.g., of the ovary. Perhaps the plug will be extruded with the next spawning event and does not degenerate at all.

4.6 General differences among pelvic brooding species

In all three pelvic brooding species of ricefishes, the developing eggs remain attached to the female via long attaching filaments. In *O. eversi* and *O. sarasinorum,* the filaments are anchored in the plug, which is formed during brooding (Hilgers et al., 2022; Iwamatsu et al., 2008). However, minor differences are obvious in the description of plug formation in *O. sarasinorum*, such as the location (ovarian cavity versus gonoduct), the identification of the interstitial cells as epithelial cells, or the description of the urogenital pore in *O. sarasinorum* (see Iwamatsu et al., 2008) versus a genital opening completely separated from the urinary duct and its opening in *O. eversi*. It also appeared striking to us that no multinucleated giant cells are reported for the plug of *O. sarasinorum*, typical for a foreign body granuloma. Different

formations of the plug between the two congeners are unlikely, yet possible. It was also reported that the plug of *O. sarasinorum* degenerates and is released within five days after hatching (Iwamatsu et al., 2008). We thus scanned a female of this species five days after hatching and found that the plug was still present, which challenges the hypothesis that there are strong differences between the two species at least in this regard. In the third species of pelvic brooder, *A. oophorus*, the attachment structure inside the female reproductive system is unknown, yet the attaching filaments seem to emerge from the ovary (Gundo et al., 2013). Additional histological studies, including of *A. oophorus*, are therefore necessary to further understand the evolution of the ricefish plug and of pelvic brooding.

5. Conclusions

This study supports the hypothesis that the formation of the plug, a novel attachment structure in pelvic brooding ricefishes, is based on an inflammatory response. The plug is a unique transient tissue that grows in the anterior part of the gonoduct of female *O. eversi*, develops during brooding and anchors the fertilized eggs to the female. We hypothesize that the formation of the plug is similar to a foreign body reaction triggered by the retention of attaching filaments and that macrophages were released after spawning into the gonoduct to phagocytize the filaments. The epithelioid cells are activated macrophages forming a tissue that resembles a foreign body granuloma (i.e., the plug) around the filaments (the foreign body). The presence of multinucleated giant cells confirms this. Structural changes of the ovarian wall, the papilla, or the gonoduct and an interruption of oocyte maturation likely facilitate prolonged egg-carrying in the pelvic brooding species *Oryzias eversi*.

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Figure legends

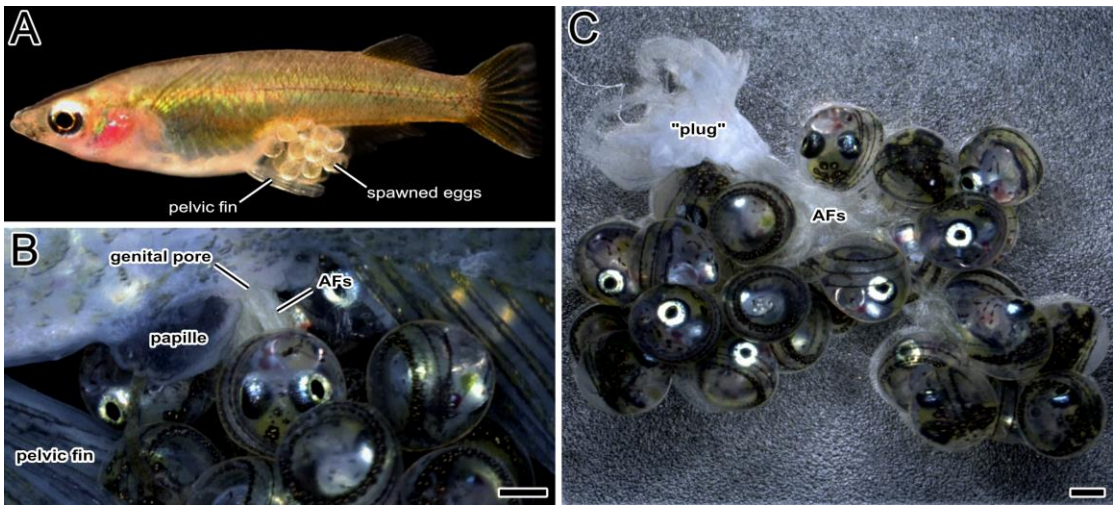

Figure 1: *Oryzias eversi*, pelvic brooding. A: A female carrying recently spawned, fertilized eggs. B: Close-up of the ventral concavity of a female 13 days after spawning. Like in all pelvic brooding species, the developing embryos stay attached to the female via long attaching filaments (AFs), which emerge from the genital pore behind the genital papilla. C: Inside the female the ends of the attaching filaments form the so-called "plug" (here, an example of the excised plug of the female in Figure 1B). Scale bars: 500µm.

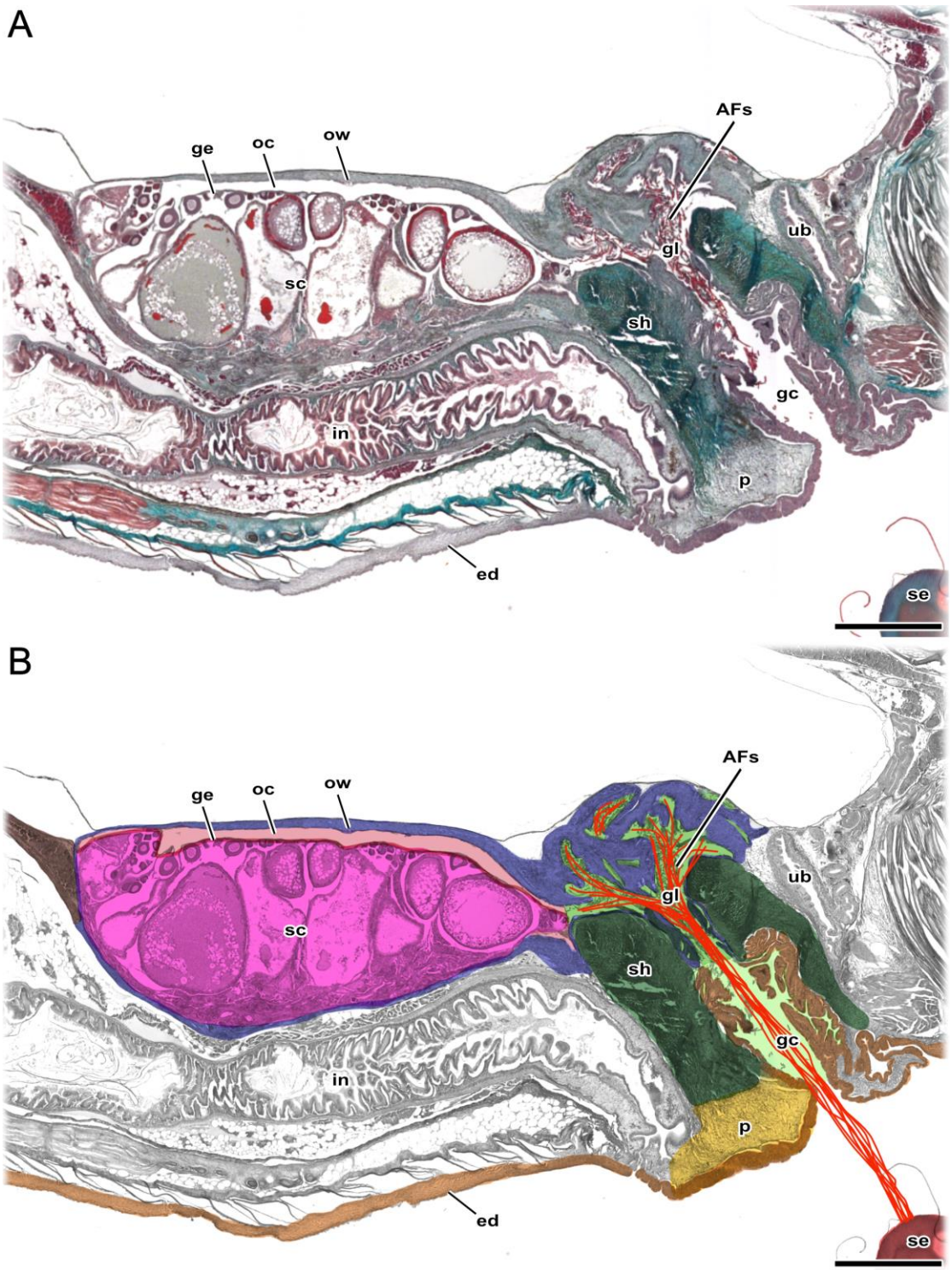

Figure 2: *Oryzias eversi*, longitudinal section of the reproductive system. A: Masson-Goldner trichrome staining. B: Superimposed colors indicating major structures. The stromal compartment of the ovary (sc) contains various oocytes and is covered dorsally by the germinal epithelium (ge). The ovarian wall (ow), surrounding the ovary, continues posteriorly. The absence of the germinal epithelium marks the transition from the ovarian cavity (oc) to the anterior part of the gonoduct, the gonoduct lumen (gl). Here, the ends of the attaching filaments (AFs) of the spawned eggs (se) are located. The posterior part of the

gonoduct, the genital cavity (gc), is formed from a modified epidermis (ed). Abbreviations: in = intestine; p = genital papilla; sh = sphincter-like structure; ub = urinary bladder. Scale bars: 500µm.

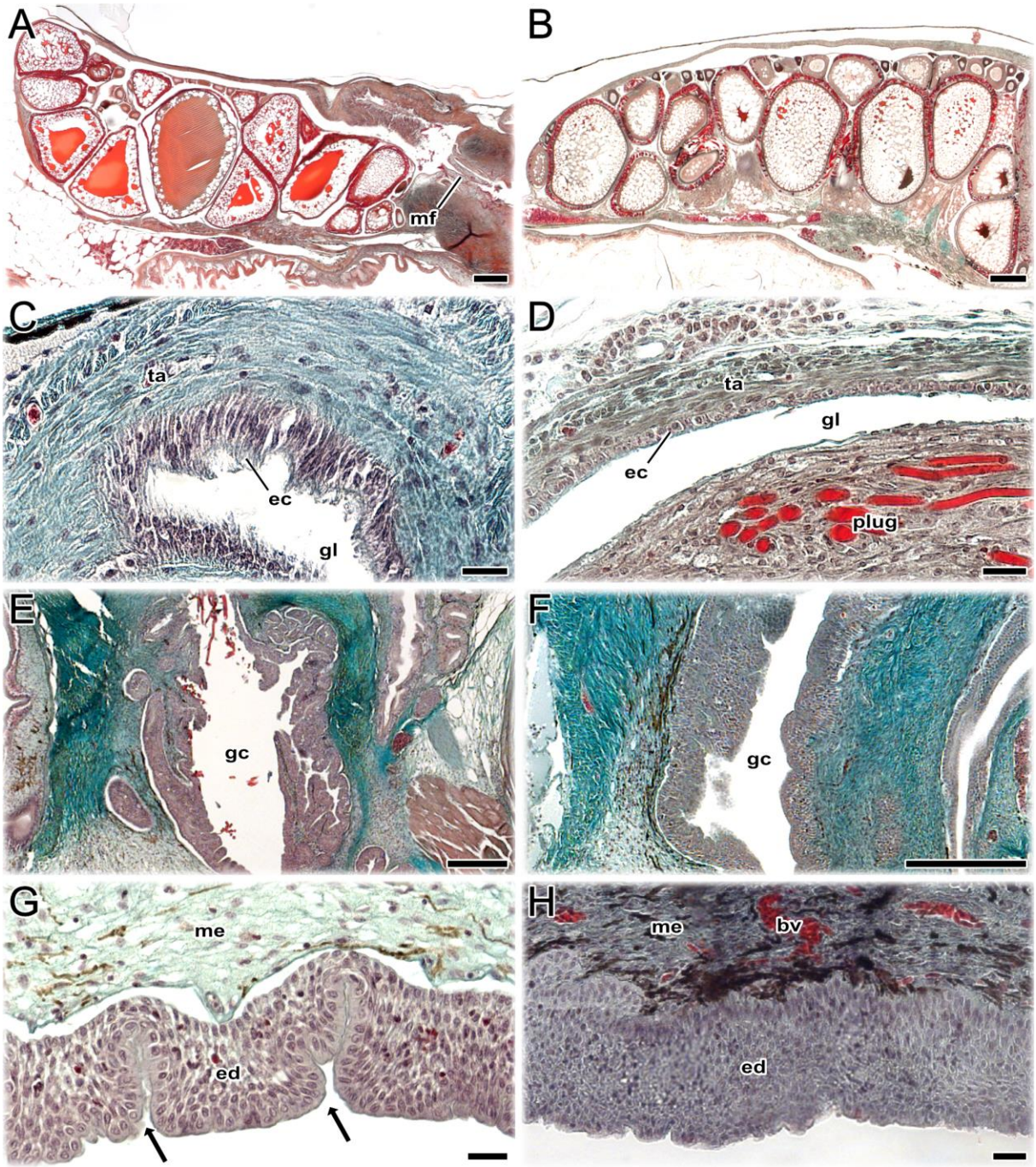

Figure 3: *Oryzias eversi*, changes in the female reproductive system during the brooding cycle. Masson-Goldner trichrome staining, longitudinal section, 5 µm. Left side (A,C,E and G) = brooding stages and right side (B,D,F and H) = non-brooding stages. A–B: The changing oocyte composition of the ovary. C–D: Micro- anatomical changes of the gonoduct lumen

(gl). E–F: Changes in the structure of the genital cavity (gc). G–H: Changes of the papilla (ed). G: After spawning invaginations (arrows) were observable. Abbreviations: bv = blood vessel, ec = epithelial cells, me = medulla, mf = mucosal folds, ta = tunica albuginea. Scale bars: A, B, E, and F = 200µm; C, D, G, H = 20µm.

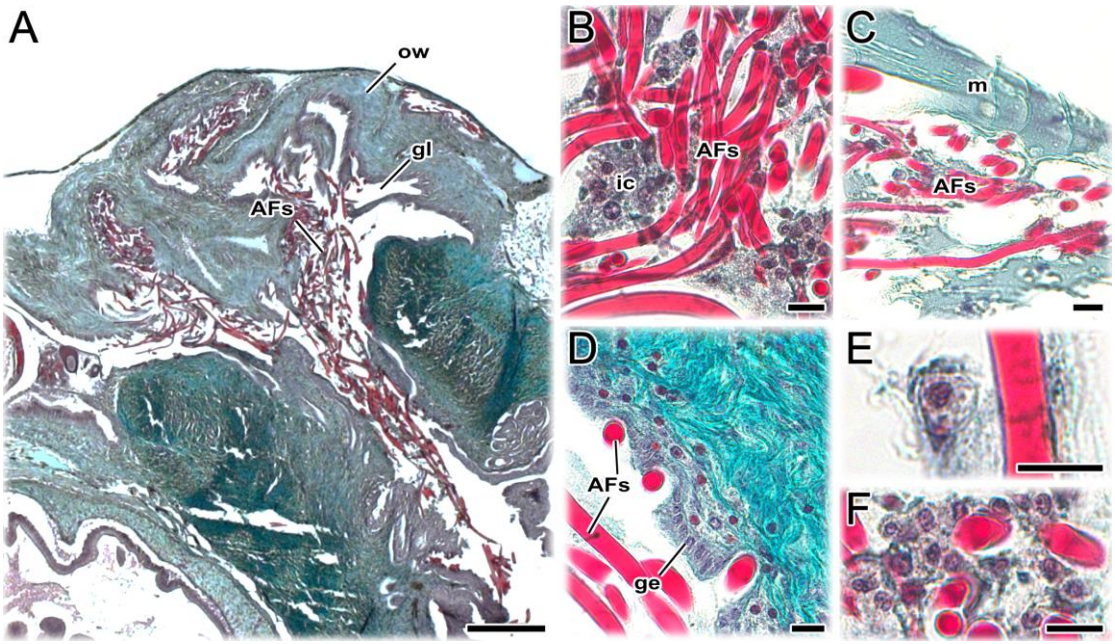

Figure 4: *Oryzias eversi*, micrographs of histological, longitudinal sections of the developing plug of a female one day after spawning, Masson-Goldner trichrome staining, 5 µm. A: The ends of the attaching filaments (AFs) closely filled the intricate recesses of the ovarian wall (ow) of the gonoduct lumen (gl). B: The filaments are loosely entangled with each other and some interstitial cells (ic). C: A non-cellular structure, probably mucus (m) is present. D: The attaching filaments puncturing the gonoduct epithelium (ge). E: Some interstitial cells were separated and larger. F: Others grouped together along the filaments. Scale bars: A = 200µm; B–F = 10µm.

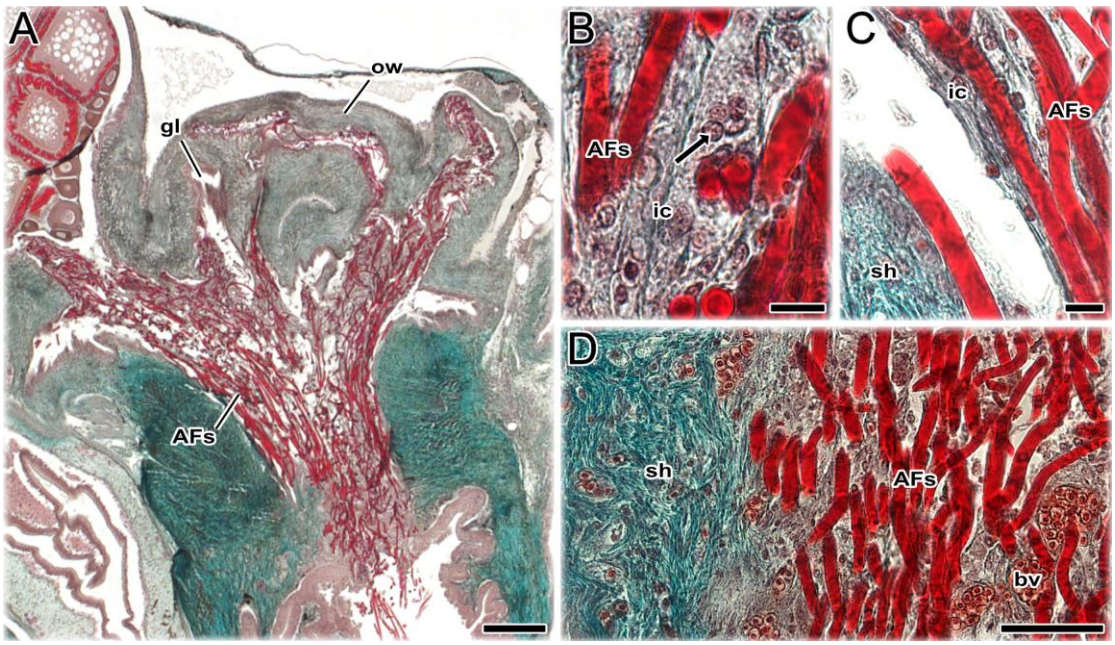

Figure 5: *Oryzias eversi*, micrographs of histological, longitudinal sections of the developing plug of a female seven days after spawning, Masson-Goldner trichrome staining, 5 µm. A: The attaching filaments (AFs) are entangled in the gonoduct lumen (gl) between protuberances of the ovarian wall (ow). In the ovary vitellogenic oocytes of the stage 5 but not fully-grown oocytes can be seen. B: The number of interstitial cells (ic) is increased, and some cells appear to start to fuse together (arrow). C: Thinner and elongated cells surround the developing tissue. D: The posterior part of the plug tissue is traversed by blood capillaries (bv) developed from the surrounding sphincter-like structure (sh). Scale bars: A = 200µm; B and C = 10µm; D = 50µm.

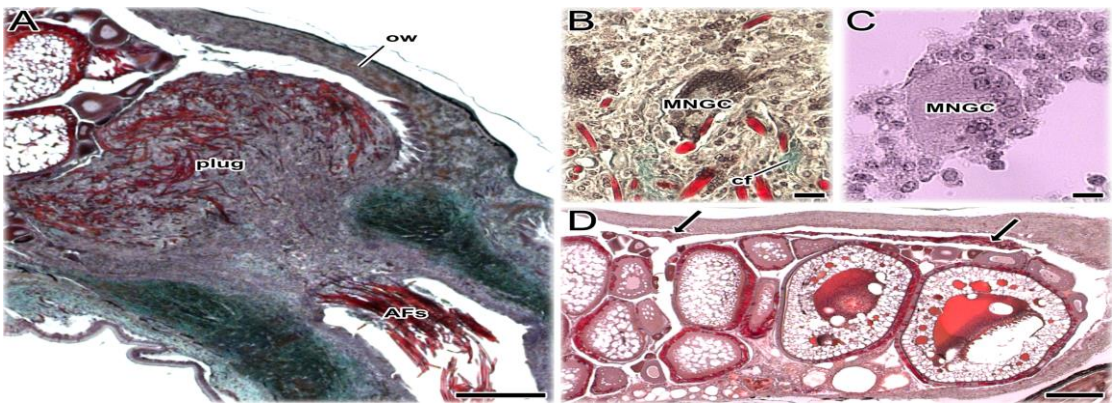

Figure 6: *Oryzias eversi*, micrographs of histological, longitudinal sections of the developing plug of a female one day after hatching of the fertilized eggs, Masson-Goldner trichrome staining, 5 µm. A: The developed plug-like structure is fused to the inner wall of the anterior part of the gonoduct. The ovary contains vitellogenic oocytes. B+C: Variation of types of

multinucleated giant cells (MNGC) in the plug. D: In one specimen, the plug extends above the ovary into the ovarian cavity (arrows). Within the stromal compartment of the ovary, large vitellogenic oocytes with yolk spheres were seen. Abbreviations: AFs = attaching filaments, cf = collagen fibrils, ow = ovarian wall. Scale bars: A and D = 200µm; B and C = 20µm.

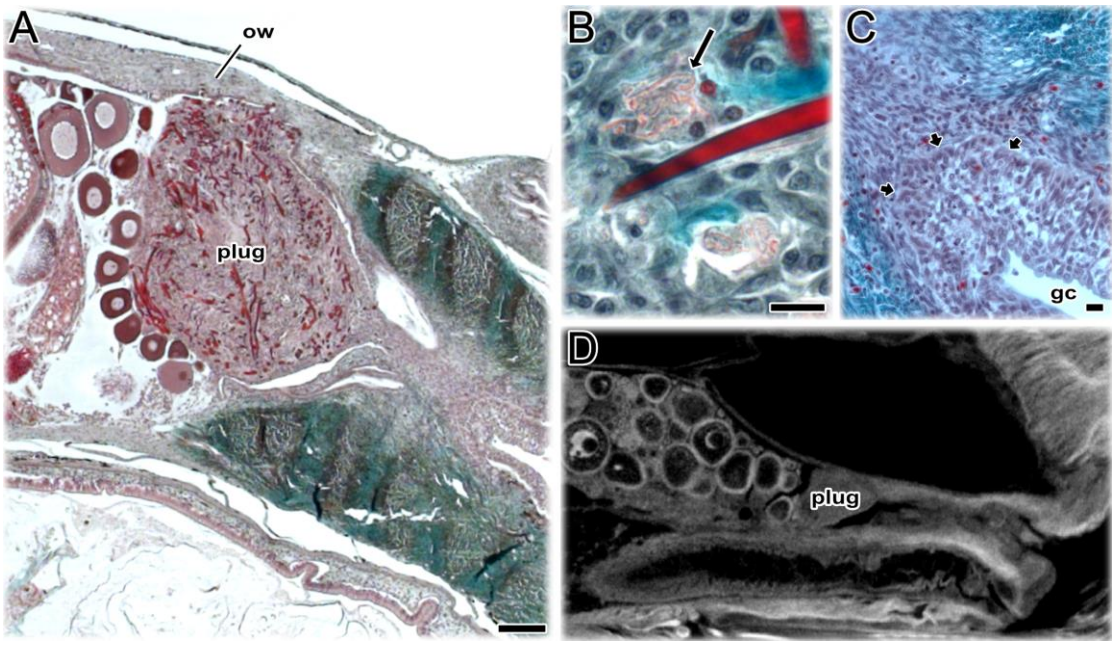

Figure 7: *Oryzias eversi*, degeneration of the plug of a female. A–C: Micrographs of histological, longitudinal sections three days after the loss of filaments, Masson-Goldner trichrome staining, 5 µm. A: The plug is still fused to the inner wall of the anterior part of the gonoduct. No vitellogenic oocytes could be observed. B: Additional, light red, thin threads are observed inside the plug (arrow). C: The interstitial cells forming the plug resemble epithelial cells in coloration, but the border between the interstitial cells of the plug that fused with the anterior part of the gonoduct and the subsequent epithelial cells of the posterior part of the gonoduct is clearly recognizable (short arrows). D: µCT-scan of a female 15 days after the loss of filaments. The plug is still present, but seems to be less fused. Abbreviations: gc = genital cavity, ow = *ovarian* wall. Bar: A = 100µm; B and C = 10µm.