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To cite this article: M A Firmansyah *et al* 2021 *IOP Conf. Ser.: Earth Environ. Sci.* **919** 012062

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Observation on the reproductive behavior and embryo of the daisy's ricefish, *Oryzias woworae* in laboratory condition

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Abstract. *Oryzias woworae* is an endemic ricefish from Muna Island, Southeast Sulawesi, traded as an ornamental fish. Information on the biology of these fish is very lacking, including their reproductive. These fish also face the threat of population decreasing in the wild. The research aimed to examine the spawning behavior and embryonic of *O. woworae* as the basis for aquaculture. Fish spawning was carried out using 1 male: 1 female ratio, which was repeated three times. The observed spawning behavior was pre-spawning, mating process, egg number, and morphology. Behavioral observations were carried out for five days of spawning. Observation of embryos was carried out to see the stages of embryo development until hatching. The results obtained on pre-spawning behavior were changes in body color and blackened fins in males, which did not occur in female fish. Males actively swim by approaching their dorsal and anal fins while chasing females. The female releases the eggs and is attached to the substrate, rapidly fertilized by the male fish. There is a phenomenon of females carrying eggs on the genital pore before being released to the substrate (non-pelvic brooder). Spawning only occurs during the day, and spawning is getting faster, which is at 09:00 AM on the fifth day. *Oryzias woworae* eggs have a diameter of 1.3 ± 0.2 mm, equipped with attaching and non-attaching filaments. Observations of *O. woworae* embryos showed that embryo hatching occurred at seven days 23 hours post-fertilization.

1. Introduction

Oryzias woworae is a species of ricefish endemic to freshwater on Muna Island, Southeast Sulawesi [1]. Morphologically, male fish has a blue color with reddish fins, while females have a silvery yellow color [1, 2]. The beauty of its color and its endemic status is the uniqueness of this fish so that it has been traded as an ornamental fish in the domestic and foreign markets. However, this economic potential has not been supported by the availability of cultivation information, so that it still relies on natural catches. Therefore, the population of these fish in nature has decreased and is threatened with extinction [3]. Based on this, research on *O. woworae* aquaculture needs to have been carried out to support production as an ornamental fish and its conservation.

Fish farming requires basic information to support the success of mass fish production in a controlled system. The information includes reproduction and early development of the life cycle.



Knowledge of reproductive aspects is very important to support the success of fish spawning. This aspect is related to synchronization at the right time and place for spawning [4] this relates to the interaction between male and female broodstocks as well as the right time and suitable places for spawning. Spawning synchronization can be seen through pre-spawning behavior [5, 6, 7], body-color changes [8, 9], courtship behavior, fertilization methods, and laying eggs [5, 6, 7]. In aquaculture activities, this becomes the basis for broodstock pairing, substrate laying, and egg harvesting [6]. Furthermore, to obtain larvae used for seed production activities, knowledge of the early development of fish life in the embryonic phase is very important for aquaculture. Observation of embryos was carried out to obtain information on the stages of embryo development until hatching [10, 11] so that it became the basis for a suitable egg incubation method.

Knowledge of reproductive behavior and fish embryos for aquaculture is essential. In the early stages, this information is needed for laboratory-scale fish rearing [12]. Therefore, the research aimed to observe pre-spawning behavior, spawning process, egg pattern released during spawning (sexual reproduction and type of laying eggs), number of eggs produced, egg morphology, embryo development under controlled laboratory conditions.

2. Methods

The research was carried out in March 2021 at the Aquaculture Laboratory, University of Sultan Ageng Tirtayasa. Broodstocks of *Oryzias woworae* was obtained from an ornamental fish supplier in Depok, West Java, Indonesia. The breeding activity was conducted in triplicates. Each pair of broodstock is put in a spawning aquarium. There are three aquariums, measuring 30 cm × 30 cm × 30 cm with a water depth of 25 cm. Slow aeration was provided to each spawning aquarium. The spawning substrate uses smoothed raffia [6]. The behavior of spawning fish was observed for five days. During spawning, the fish were fed with dry *Artemia* (polar red). Feed was given twice a day at satiation. During spawning, siphoning and water quality measurements (temperature, pH, and dissolved oxygen) are carried out. Observation of embryos using 3 eggs. Incubate eggs using a 450 ml container. Egg incubation containers are not equipped with aeration. Embryo development was observed using a microscope. The embryonic developmental stages observed were cleavage, morula, blastula, gastrula, and organogenesis until hatching. During egg incubation, water quality measurements were taken, namely temperature, pH, and dissolved oxygen. Data on reproductive behavior and embryos of *O. woworae* were analyzed descriptively.

3. Results

3.1 Spawning behavior

The pre-spawning activity of *O. woworae* does characterized by the male movement, namely their dorsal and anal fins that are displaying to attract females for spawning. In addition, the body color of males that will spawn looks stronger, namely metallic blue and slightly blackened on the dorsal fin, anal fin, and caudal fin (Figure 1). Color changes during spawning did not occur in female *O. woworae*.

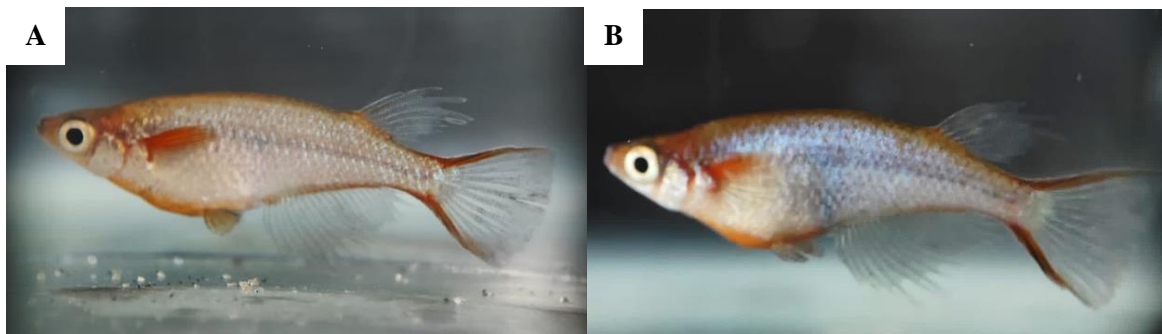


Figure 1. Changes in body color in male *Oryzias woworae* before breeding mood (A) and during breeding mood (B).

During spawning *O. woworae*, i.e., male parent fish chased and followed the female fish closer to the substrate. The eggs ovulated by the female are then fertilized by the male externally and laid on the substrate by the female (Figure 2.A). The observation found that the behavior phenomenon of female broodfish carrying fertilized eggs in the urogenital part just before being placed on the substrate (Figure 2.B). The spawning behavior of male and female *O. woworae* fish only occurs during the day and is not seen at night.

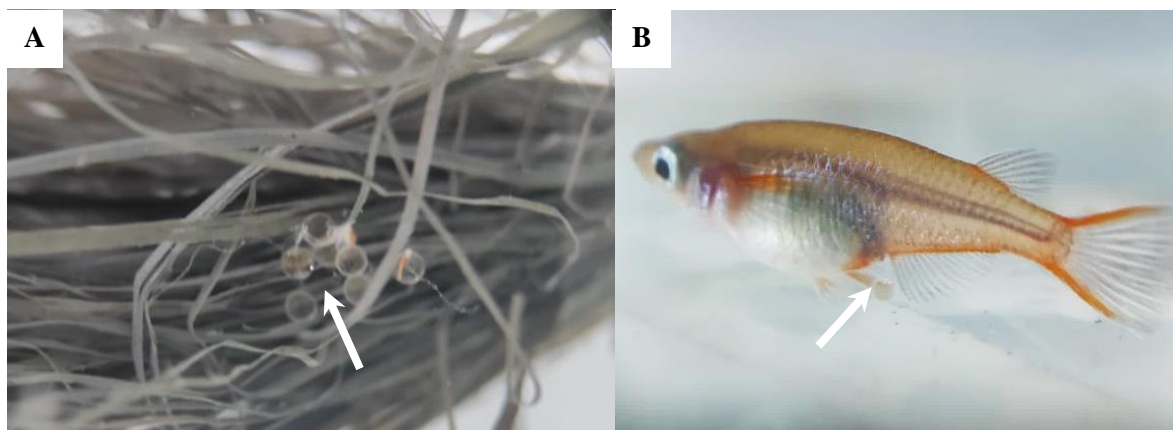


Figure 2. *Oryzias woworae* eggs are attached by female fish on the substrate (A) and female *O. woworae* carry eggs on the urogenital (B).

The synchronization pattern of male and female *O. woworae* in spawning can be seen based on the percentage of eggs produced during five days of spawning. Spawning synchronization did not occur on the first day after *O. woworae* was put into the spawning container. *Oryzias woworae* began to spawn on the second day, namely in the afternoon. After the 3rd day, the synchronization of spawning accelerated, namely in the morning based on the pattern of eggs released by female fish (Figure 3).

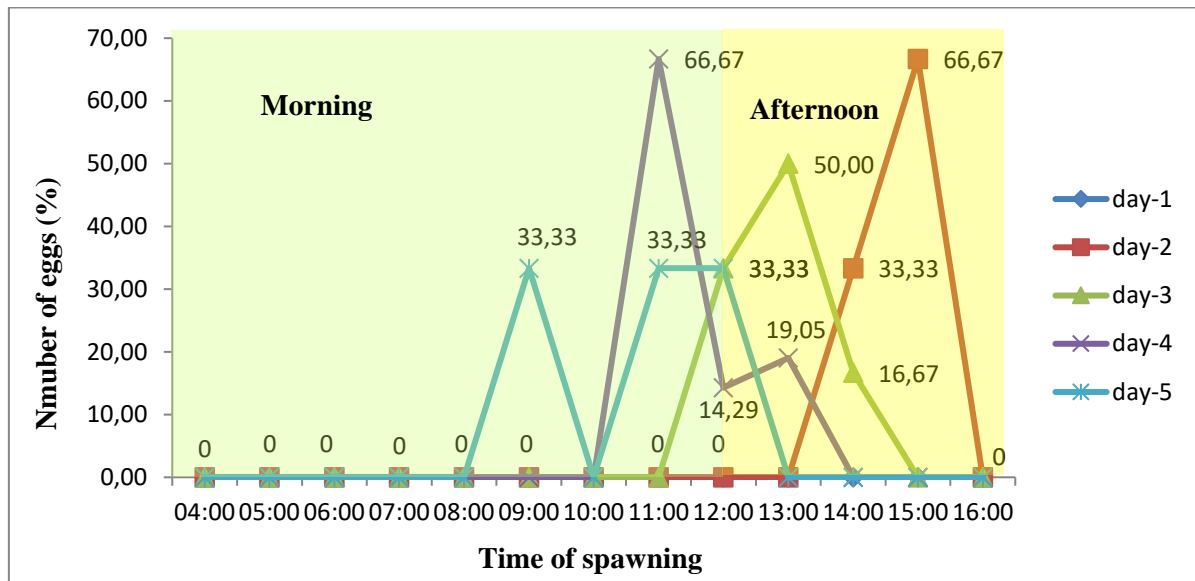


Figure 3. Spawning synchronization of *Oryzias woworae* based on the number of eggs per hour on spawning days for five days.

The average number of eggs per day produced by one female *O. woworae* ranges from 2-9 eggs. At the beginning of the fish spawning two eggs. The peak number of eggs produced was on the 4th day after the brood insert into the spawning container (Figure 4).

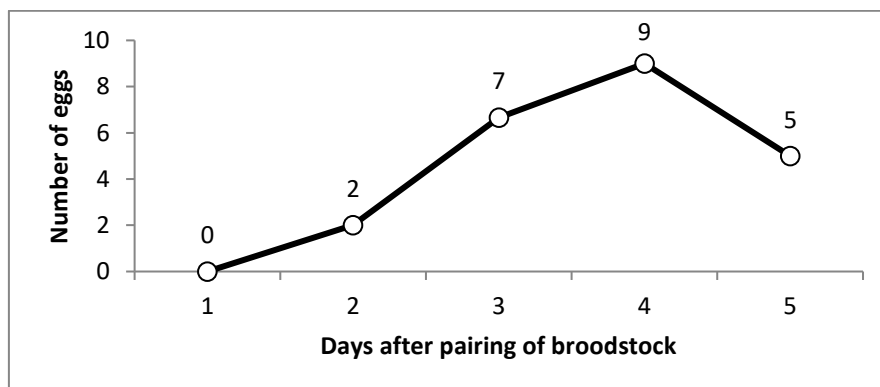


Figure 4. The average number of eggs per day from one female *Oryzias woworae* for five days of spawning.

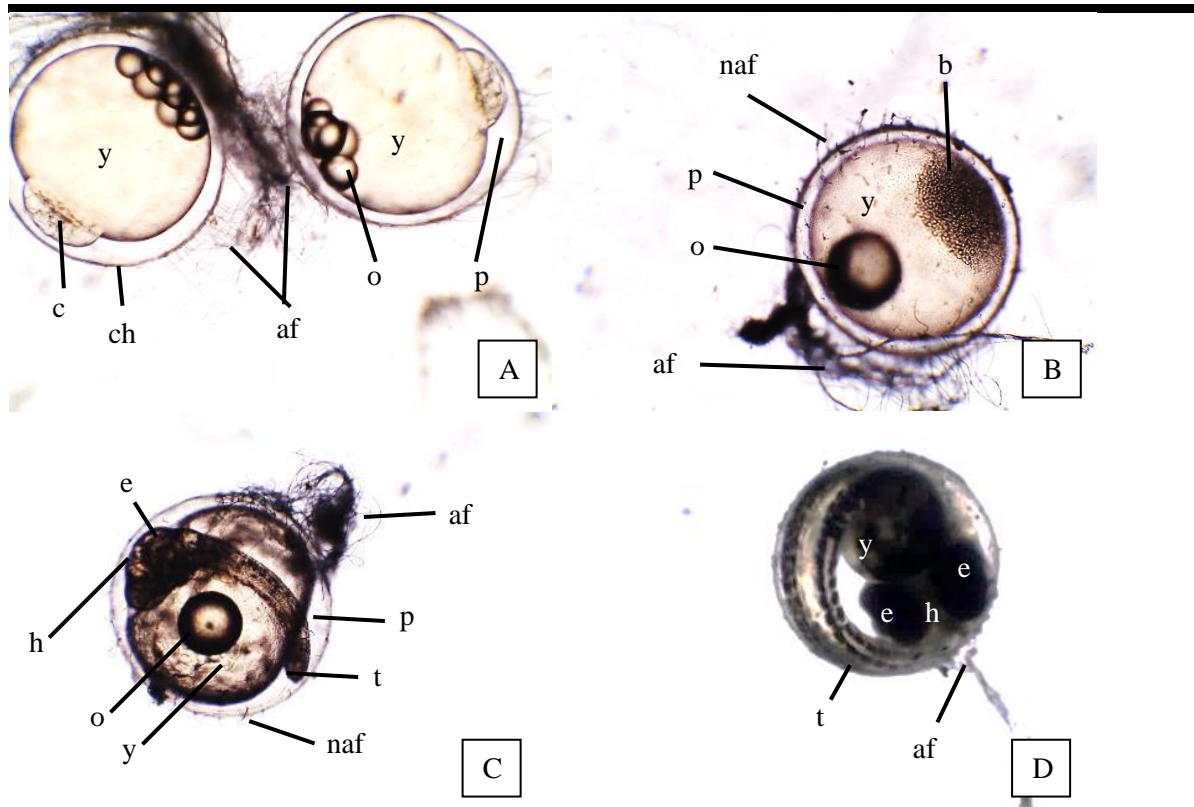
3.2 Embryo Development

Oryzias woworae eggs have a diameter of 1.3 ± 0.2 mm, which is shape round equipped with a filament that allows the eggs to stick to the substrate (attaching filament). These filaments are located around the animal poles. In addition, there are shorter filaments scattered over the chorion. These short filaments have no function to stick to the substrate, so they are called non-attaching filaments. *Oryzias woworae* embryo development occurs after fertilization. Fertilized eggs have a macroscopic clear-orange morphology, while unfertilized eggs are white. Newly fertilized eggs have large yolks and oil droplets scattered in the egg. Some characteristics of the early development of *O. woworae* fish embryos are the oil droplets moving towards the animal pole along with the embryogenesis process and the number of cells that continue to divide at the vegetal pole. At the stage of organ formation, the visible characteristics are smaller yolk size, darker eyes, more body

pigmentation, and more active embryo movements (Table 1 and Figure 5). Incubation of *O. woworae* eggs to hatch takes more than seven days at an incubation temperature of 27±2°C.

Table 1. Embryo development of *Oryzias woworae* at an incubation temperature of 27±2°C.

Stadia	Observation Time	Microscopic Description
Cleavage	1 hours 30 minutes	Oil globules are evenly distributed in the egg and tend to move towards the vegetal pole.
Morula	8 hours 33 minutes	The size of the dividing cells is getting smaller, and the number is increasing, which is more than 32 cells. Oil globules have gathered at the vegetal pole as much as 2-6.
Blastula	12 hours 25 minutes	The blastodisc is visible at the animal pole. In this phase, 1-3 oil globules are visible.
Gastrula	14 hours 4 minutes	The blastoderm begins to cover half of the yolk and moves towards the vegetal pole, where the oil globule location.
Mid-embryo (Organogenesis)	86 hours 25 minutes	The head and tail can be distinguished, the eyes have been formed but do not have pigment, the yolk size is small, the heart and blood vessels are visible.
Late embryo (Organogenesis)	152 hours 3 minutes	The yolk size is getting smaller, the eyes are getting darker, the pectoral fins are visible, and the embryo's movements are more activated.
Hatching	190 hours 39 minutes	Larvae



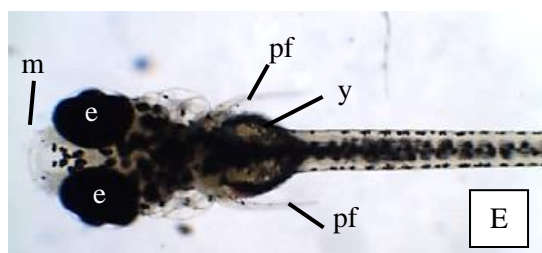


Figure 5. The early life of *Oryzias woworae* at an incubation temperature of $27\pm 2^{\circ}\text{C}$. Morula (A), Blastula (B), Mid-embryo (C), Late embryo (D), Larvae (E). af: attaching filament, b: blastodisc, c: cells, ch: chorion, e: eye, h: head, m: mouth, naf: non-attaching filament, o: oil globule, p: perivitelline space, pf: pectoral fin, t: tail, y: yolk.

Results of water quality measurements during the study known value of water quality is still optimal for fish spawning and egg incubation *O. woworae* (Table 2).

Table 2. Results of water quality measurement.

Water quality parameters	Spawning	Egg incubation
Temperature ($^{\circ}\text{C}$)	26.5 – 31.0	26.0 – 31.0
pH	7.8 – 8.5	7.7 – 8.8
Dissolved Oxygen (mg L^{-1})	5.4 – 6.0	5.3 – 5.9

4. Discussion

4.1 Spawning behavior

Oryzias woworae spawning begins with changes in behavior and color in male fish in pre-spawning. It has also been reported in spawning *O. dancena*. Male fish actively pursue and try to stimulate female fish to spawn [5]. In species *O. soerotoi*, breeding males fight each other and court females by displaying their black bodies and fins [8], and the same behavior occurs in males *O. dopingdopingensis* with dark brown to black bars [9]. The behavior of males displaying the dorsal and anal fins also occurs in *O. hadiatyae* [13]. The phenomenon of sexual dimorphism in male fish is more dominant in tropical fish. In addition, male parents are more aggressive in spawning [7]. It indicates that the male individual of the *Oryzias* species is more active in initiating the mating process. During the intimidation process, pheromones released by the male parent against the female parent can stimulate the female parent to spawn [14].

Activities spawning in *O. woworae* indicate that this fish is a type of external fertilization. After fertilization of the female lays an egg on a substrate or a moment seen carrying eggs that have been fertilized in the urogenital before it is placed on a substrate, this is similar to the fish spawning *O. dancena*, i.e., male and female, simultaneously with the fertilized eggs in the urogenital [5]. The behavior of laying eggs on the substrate shows that *O. woworae* is a type of rice-fish that is an egg depositor or non-pelvic brooder. Reproduction of egg depositors is a common strategy in *Oryzias*. There are only two species of *Oryzias* reported as pelvic brooder i.e., *O. sarasinorum* [15] and *O. eversi* [16].

The spawning pattern associated with egg production every hour and continued for four days indicates that *O. woworae* are partial spawners. The occurrence of spawning in these fish takes one day after the installation of male and female fish. The right time and place conditions induce male and female fish synchronizing to spawn [4]. *Oryzias woworae* spawning, which only occurs in the afternoon between 09:00-15:00, is thought to be influenced by light and water temperature. This daily spawning pattern is similar to that of *O. latipes* which begin to spawn in the morning [17] between 07:00 AM and 12:00 AM, which is influenced by different environmental stimuli (temperature and

photoperiod) [18]. Spawning patterns related to the time the eggs are released can be the basis for managing egg harvest in aquaculture activities.

4.2 Embryo development

The egg morphology of *O. woworae* is similar to that of ricefish in general, such as *O. latipes* [10] and *O. javanicus* [19]. The development of *O. woworae* eggs after fertilization is influenced by water temperature [20] and salinity [21]. The early phases of the development are cleavage which is a rapid process of embryonic development from one cell divided into two cells, up to more than 32 cells [10].

After cleavage, the egg is in the morula phase, characterized by cells like piled balls (round). That is due to continuous cell division, so that it is similar to grapes sticking together tightly (Figure 5.A). The next phase, blastulation, occurs after the continued formation of the morula, which continues to divide. The onset of cell changes characterizes the shape of the blastula by holding irregular indentations (Figure 5.B). The blastula is a phase that begins with more and more blastomeric cells. In the late blastula, the blastodisc looks flatter [4]. The subsequent embryonic development is the gastrula. This phase is characterized by a thickening of the outer yolk wall of the blastodisc, which forms a semicircle in the form of a ring called the germ ring [10].

The embryo's body characterizes organ development in the embryo or early organogenesis will be seen from the eyes, head and body [10]. The end of the organogenesis phase can be marked with a visible body shape and movements of embryos increasingly active. The eye shape that develops early in the embryo is related to the initial organs needed by the larva to eat (external feeding). During embryogenesis, the energy used is derived from the yolk. Therefore, as the developmental phase approaches hatching, the yolk size gets smaller.

Oryzias woworae eggs have a long incubation time until they hatch (7 days 23 hours at 27°C). It is common in *Oryzias* egg depositors. For example, *O. soerotoi* species hatch after 14 days of incubation at 25°C [8], 11-13 days in *O. dopingdopingensis* eggs at 26°C [9]. In *Oryzias* pelvic brooder *O. eversi*, females carry eggs until they hatch after 18-19 days at 24-27°C [16]. The development is so complicated and takes a long time in the embryogenesis process, causing the larvae of *O. woworae* to have been actively swimming at hatching (Figure 5.E). *Oryzias woworae* larvae measure 4 ± 0.5 mm TL.

5. Conclusion

The conclusion of this study, *Oryzias woworae* spawning begins with body-color changes and courtship behavior in male broodstock on pre-spawning. The synchronization spawning behavior of *O. woworae* broodstocks only occurs during the day and is not seen at night. The synchronization of spawning accelerated, namely in the morning. *Oryzias woworae* eggs have a long incubation time. This development is so complicated and takes a long time in the embryogenesis process. The larvae of *O. woworae* at hatching have been actively swimming.

Acknowledgments

This research is supported by the Aquaculture Laboratory Department of Fisheries Science, University of Sultan Ageng Tirtayasa. We thank to Ilham Septian, Fani Safitri Agatha, Nisa Nafiyanti, Roy Satriadi, Crisnawati, Edo Ahmad Solahudin and all those who have helped during this research.

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