Can *Oryzias Celebensis* Embryo be Transported Dry?

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Abstract. Embryos of the genus *Oryzias* have long been used as sentinel organisms in ecotoxicological research. Compared with animal models from mammals, *Oryzias* embryo offers several advantages such as being cost-effective, more sensitive, rapid and produce very little waste. In ecotoxicological studies, it is necessary to have inter-laboratory calibration on used techniques between one laboratory and another, so that the used techniques are reliable. Inter-laboratory calibration between laboratories requires transferring embryos from one laboratory to another. For this purpose, research has been carried out to compare the survival of embryos reared in water and non-water (dry) media until they hatch. The results showed that the embryos reared with dry media hatched one day faster than those raised in water media. The dry-incubated embryo also had an average total length longer than those incubated with embryo rearing media (ERM). In this study, it was concluded that fish embryos of *Oryzias celebensis* could be transported dry for up to five days.

1. Introduction

Fish of the genus *Oryzias*, especially the species *Oryzias latipes* are the organisms most frequently used in biological experimentation for more than a century [1]; [2]. Several factors stimulate the use of the genus as a model organism[3]; [4]. For example, it is small in size [5], so the operational costs of the experiment are relatively cheap and easy to maintain. Due to its small size, very little waste is produced by the test system using *Oryzias* fish. Fish of the genus *Oryzias* can be distinguished between males and females morphologically or have external sexual dimorphism [5]; [6]; [7]. This makes it easier for us to choose male or female fish if laboratory testing requires distinguishing between males and females. Their genome size is about 20 to 40% of the mammalian genome, making them the only vertebrates available for large-scale mutagenesis [8]. The maturation time is only 2 - 3 months, which is relatively less labor-intensive and saves time to produce transgenic lines [8]; [5]. *Oryzias* fish are also easily adapted to laboratory conditions [5].

Compared to the Japanese medaka fish or *Oryzias latipes*, *Oryzias celebensis* is still rarely used as a model organism for biological research and its derivatives. A search using the keywords "*Oryzias latipes* model fish" on Google Scholar showed there were 22,200 papers. However, if we use the keywords "*Oryzias celebensis*" there are only 106 papers. These data indicate that research on *Oryzias fish* as a model fish in biological research and its derivatives such as in the field of ecotoxicology needs to be improved.

In addition to adult fish, the embryo of *Oryzias celebensis* is also very potential as a model organism in ecotoxicology research. Compared to adult organisms, fish embryos have several
advantages such as their very small size. The diameter of *Oryzias* embryos is about 1.5 mm. Because of its small size, laboratory testing using *O. celebensis* embryos can use small containers such as microplates. Compared to other fish eggs, *Oryzias* eggs are large and transparent [8]; [9]. This makes it easier for us to observe the dynamics of embryo development without killing the embryo. From the perspective of biomarker studies, egg transparency is a very useful factor for developing non-directive biomarkers [10].

To produce a good protocol for the use of *O. celebensis* embryos as a model organism, inter-laboratory calibration is necessary. In this process, it is necessary to send specimens from one laboratory to another. During the rearing of *O. celebensis* broodstock in the laboratory, we often encounter unharvested *O celebensis* eggs in dry parts of the aquarium, like sticking to the dry part of the pump. After being observed, it was evident that the embryos in the eggs were still alive and developed until they hatched. From this experience, we hypothesized that *O. celebensis* embryos could be incubated under waterless conditions and could therefore be transported dry. This paper is discussing the results of research on embryos of *O celebensis* which were incubated dry and using embryonic rearing media (ERM) for inter-laboratory calibration for ecotoxicological studies.

2. Material and Methods

2.1. Chemical

The chemicals used in the study were embryo rearing media (ERM) (10.0 g NaCl, 0.3 g KCl, 0.4 g CaCl₂·H₂O, 1.63 g MgSO₄, 1 ml of NaHCO₃ (0.25 g/20 ml H₂O)) which purchased from Merck, Germany.

2.2. Eggs production

Some pairs of *Oryzias celebensis* were put into the aquarium with 20 liters of water (Figure 1). The fish were incubated until they produce eggs. The fish were fed with *Artemia* sp nauplii and commercial feed Feng Li during the incubation process.

2.3. The experiment

After two months of being fed with *Artemia* nauplii and commercial feed Feng Li, the broodstocks begin to produce eggs. The broodstocks of *Oryzias celebensis* carry fertilized eggs in their abdomen. The attachment between one egg and another on the abdomen is assisted by threads produced by the female broodstock (Figure 2). Because the eggs were held together by threads, after being released from the female broodstock abdomen, the eggs were put into a petri dish that has been filled with ERM (Embryo Rearing Media) solution to be separated. The egg collection was gently pressed with the forefinger and rotated until the eggs were separated from one egg to another. Once separated, the eggs were put into 24 microplate wells individually to be carried out as model organisms in the experiment.

![Image](image_url)
Figure 1. Broodstock of *Oryzias celebensis*. The red circle is a sign of a male, and the blue circle is a sign of a female.

Figure 2. Broodstock of *Oryzias celebensis* carries eggs in the abdomen. Inside the red circle are the eggs.

The experimental design used was a completely randomized design (CRD) with two treatments, incubation with ERM and dry incubation (incubation without water medium), with four replications. Eggs that were given dry treatment still received water when they were observed on each day of observation. This is similar to the condition of eggs in the riverbank which is still receiving splashes of river water. Eggs that experience dry conditions on riverbanks are similar to eggs that experience metabolic dormancy [11].

Embryos were incubated until they hatched. Embryos were given dry treatment hatched on the fifth day after fertilization. The hatched larvae were immediately measured for total length. In general, *Oryzias* embryos hatch on days 8-10 after fertilization[12];[3]. In the present experiment, embryos incubated with ERM hatched on day 8.

2.4. Data analysis
Student t-test was used in this study to compare data from the two treatments. The requirements in the Student t-test have been met, the data are normally distributed and homogeneous.
Figure 3. The fertilized *Oryzias celebensis* embryo in stage 5. The egg diameter was 1.32 mm (magnification 40 x).

Figure 4. Experimental design

3. Results and Discussion

*Oryzias celebensis* produces eggs in average fertilization of 25-30, but in our laboratory, this fish can produce up to 80 eggs. The eggs used in this study were produced by one broodstock. Not all *Oryzias* eggs are the same color. *O. celebensis* and *O. javanicus* eggs are white (Figure 2), while *O. wolasi* eggs are clear orange in color.

On the male of *O. celebensis* fish, when the gonads are mature 4-5th (ready to mate) the body color becomes brighter and more contrasting (Figure 5). The pattern of the body lines is more contrasting when compared to those who are not ready to mate. However, if the fish is disturbed, the body color will return to its original color.

The broodstock *O. celebensis* performs a unique mating behavior as that of *Oryzias latipes*. The unique mating behavior consists of several stages, such as following, dancing, floating, Crossing, and Separation [13]. In the crossing step, the male holds the female using dorsal and anal fin and brings her cloaca closer to the female's cloaca. Anal fins in males which are larger than females have a significant role in the process of fertilization [14]. After that, the two broodstocks move down while vibrating their bodies for a few seconds while releasing eggs and sperm [13]. Fertilized eggs are deposited in the abdomen of the female broodstock with fine threads growing from the outer layer of the egg (Figure 2 and Figure 3). Fertilization activity usually occurs at 08-00 -14.00.

Figure 5. Male *Oryzias celebensis* ready to mate [15]

Not all eggs are fertilized in the external fertilization process that occurs in *Oryzias* fish. Some eggs are fertilized (active) and some are not (inactive). The presence of Ca ions and pH greatly affect the activation process of *Oryzias* eggs [16]. The presence of a perivitelline space is a sign of a fertilized egg (Figure 3). In nature, the genus *Oryzias* may undergo metabolic dormancy in which eggs or embryos are laid by broodstock in riverbanks. Although the embryos are not submerged in water and may only get a
splash of water, they are still alive and developing until they hatch [11]. This phenomenon prompted this study to obtain scientific justification in the dry transport of *Oryzias celebensis* embryos for inter-laboratory calibration in ecotoxicological studies.

The experimental results showed that both embryos incubated with ERM and dry media depicted 100% hatchability. In addition, the results of statistical tests revealed that dry-incubated embryos hatched faster than those incubated with ERM media (Figure 6). The average incubation period for embryos with dry treatment was 137.25 hours, while those incubated with ERM had an average of 199.00 hours. The shorter incubation period in the embryos incubated in dry conditions was thought to be due to the higher temperature compared to those incubated with ERM. *O. latipes* incubated at 24 °C had the longest incubation period compared to those incubated at 28 and 32 °C [17]. Incubation in dry conditions which is certainly associated with an increase in temperature may cause the process of masculinization of *O. celebensis* larvae, as happened in *O. latipes* due to increased cortisol [18]. Hence, it is necessary to conduct further research on dry-incubated *O. celebensis* to ascertain whether there is an occurrence of masculinization because it will affect inter-laboratory calibration performance in using embryos as an animal model.

The ability of *Oryzias celebensis* embryos to grow and develop in dry conditions is probably the result of the K strategy used by *O. celebensis*. K strategy refers to any of the reproductive strategies adopted by an organism, characterized by the production of very few offspring or eggs with a large investment of energy in the egg or embryo. The opposite is the R strategy in which the organism produces a large number of offspring or eggs but invests little energy in the resulting embryo [19]. Therefore, embryos of *O. celebensis* were constructed by broodstock to have a high hatching percentage and larval viability. To ensure maximum hatching and high viability of larvae, *Oryzias* broodstocks equip their embryos with a thick and hard chorion layer that can cope with various environmental stressors[13]; [20]. There are several functions of the chorion that explain why it is an important tool in the K strategy so that the *O. celebensis* embryo can live and develop in relatively dry conditions, as the following: 1. The chorion functions to attract sperm, activate spermatozoa and prevent polyspermy [21];[13]. 2. The hard and thick chorion layer protects the embryo from environmental disturbances such as dryness and does not break easily [13]. Before the chorion thickens and hardens, the embryo absorbs water which causes ultrastructural and cytochemical changes [21]. Hardening of the chorion is caused by alveolar colloids, Ca\(^{2+}\) ions, phospholipids, and enzymes in the chorion layer (glycoproteins) or enzymes found in the perivitelline space[22]; [23]. The chorion can block the diffusion of unwanted molecules so that if there is a diffusion of molecules into the embryo, the diffusion process will be very slow and minimal [21]. For this reason, the chorion is strengthened with an extraembryonic membrane layer beneath the chorion layer [20]. 4. Bacterial and similar infections can also be prevented by a thick and hard chorion layer [21]; [24].
Figure 6. The incubation period between embryos reared in ERM and dry media. There was a statistically significant difference in the average of incubation periods between those incubated with ERM and dry (p > 0.05).

Where exactly the origin of the chorion material is produced is still debated by some scientists. The chorionic layer of *O. latipes* according to Tesoriero is a product of the oocyte and the mechanism of chorion secretion involves synthesis in the cytoplasm of an organelle called the Golgi apparatus, and small, dense vesicles transfer the chorionic precursor material to the developing chorionic layer [25]. This opinion was refuted by Kinoshita and co-workers. The authors stated that biochemically the constituent substances of chorion originate from the liver [13]. This opinion is supported by Murata who confirmed that choriogenin gene expression occurs exclusively in the liver and does not occur in the ovaries [26]; [27]. Hamazaki and co-workers also mention that the glycoproteins that make up the chorion originate from the liver [28]. However, Kammori by analyzing cDNAs encoding found that *O latipes* eggshell glycoproteins originate from oocytes [29]. Finally, it can be concluded that the chorion of *Oryzias* originates from the liver and oocyte. In the development of studies on the *Oryzias* chorion, researchers have classified Choriogenin into choriogenin L [26], choriogenin H [27], and choriogenin H minor [30]. For ecotoxicological studies, choriogenin has been used as a biomarker of endocrine disruption chemicals [31]; [32].

The results showed that the total length of larvae at hatching in the embryos reared in ERM and dry media showed a statistically significant difference (p < 0.05)(Figure 7). Embryos incubated on dry media had a mean total length (mean 5.13 mm) longer than those reared in ERM (mean 4.71 mm). Dry incubation of *Oryzias celebensis* embryos mimics the metabolic dormancy that occurs in fish for protecting their offspring [11] by laying their eggs on riverbanks that are not submerged by river water. This behavior has to do with how broodstocks keep embryos away from predators. Another advantage is that the embryo is automatically protected from contaminants that can enter through diffusion. Naturally, as we know that the diffusion of certain molecules occurs in the *Oryzias* embryo which runs slowly due to the embryonic layer below the chorion [20]. If there is the diffusion of contaminants into the embryos, the embryos must use their energy to overcome the contaminants that enter by diffusion. In such conditions, the embryo loses some of its energy for the growth process. In embryos that live in dry conditions or not in a water medium, the problem of diffusion that carries pollutants or other molecules does not occur, so that the energy prepared by broodstock can be used optimally for the growth process. In addition, embryos that live in dry conditions do not receive water pressure, so the energy used to overcome water pressure can be used for the growth process. Therefore, in this study, *O celebensis* embryos incubated in dry conditions or without water had a longer mean total length than embryos incubated with ERM.
Figure 7. The difference in total length of newly hatched larvae reared in ERM and dry media. Asterix showed a significant difference between the two treatments [p < 0.05].

4. Conclusion
This study showed that dry-incubated *Oryzias celebensis* embryos and those incubated with ERM had 100% hatchability. However, dry-incubated embryos had a faster incubation period with a longer average total length than those incubated with ERM media. The results of this study also suggested that *O. celebensis* embryos could be transported dry for up to five days under slightly humid conditions.

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