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Dry transportation of *Oryzias wolasi* embryo for ecotoxicological studies

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Abstract. Fish of the genus *Oryzias* have long been used as model fish or sentinel organisms in ecotoxicological studies. Intercalibration between laboratories is fundamental in producing test procedures in ecotoxicological studies. In the inter-calibration activities, the transport of materials used in testing is important. Research on the dry transport of embryos, *Oryzias wolasi* was conducted. The test was carried out using a 24 well microplate. The parameters analyzed were the incubation period, hatching success, and the total length of fish at hatching. The experimental results showed that there were no statistically significant differences ($p > 0.05$) in the incubation and hatching success of the embryos were incubated in embryo rearing media (ERM) and those were incubated with dry incubation. However, there were significant differences ($p > 0.05$) in the total length of newly hatched larvae. The average of the larvae total length that were reared dry was longer compared to those were reared in ERM. The study suggested that the fish embryos of *Oryzias wolasi* could be transported dry for ecological studies and others.

1. Introduction

Fish from the genus *Oryzias* or known in Japan as *medaka* is a model or sentinel organism that is used in toxicological and ecotoxicological studies. This fish is used as a model organism because it is mainly small in size [1,2]. Due to its small size, if we use *Oryzias* as a sentinel organism in ecotoxicological studies we do not require a large space, a lot of water media, and of course, the experimental system will produce less experimental waste so it is easy and cheap to handle. The genus *Oryzias* also has a short regeneration time [3] so it doesn't take a long time to get the results of multiple generations experiments. On the other hand, the short regeneration times also provide multiple end-points in fish development and reproduction. The differences between male and female in *Oryzias* is very easily recognized through secondary sexual characteristics [3]. This will facilitate ecotoxicological studies involving gender as a determinant factor. In addition, fish of the genus *Oryzias* are easy to maintain and breed [1,2] at the laboratory level. The ease of gender recognition also contributes to the breeding process. Furthermore, the ease of maintenance and breeding of *Oryzias* provides convenience in adapting these fish to various experimental designs planned. The



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adult female of *Oryzias* spawns daily for 3-4 months in the laboratory thus providing a continuous supply of test specimens for various experiments. The chorion of the *Oryzias* egg is transparent so that the embryo can be observed during its development without removing the shell [4].

As part of the *Oryzias* genus, the *Oryzias latipes* distributed in Japan, China, Taiwan, and Korea [5] are the species most intensively studied [6]. Other species that have been widely studied such as *O. javanicus* [7-9], *O. melastigma* [10,11], *O. sinensis* [12,13]. In addition, the *Oryzias* which are endemic to Sulawesi such as *Oryzias celebensis* has also begun to be used as model fish in toxicological and ecotoxicological studies. Indeed, Sulawesi Island has many genera of *Oryzias*, one of which is endemic in Southeast Sulawesi, which was recently identified, namely *Oryzias wolasi* [14]. This fish is smaller than *O. celebensis* (maximum total length = 6 cm). The maximum total length of *O. wolasi* is only 2.9 cm. Therefore, *O. wolasi* is very potential as a model fish in ecotoxicological studies.

One of the important factors in establishing an ecotoxicological test protocol is conducting an inter-calibration program among ecotoxicology laboratories in Indonesia. One of the problems in doing inter-calibration in Indonesia is the transportation of specimens from one island to another. In addition to adult animals, one thing that can be used for ecotoxicological tests of the genus *Oryzias* is eggs containing embryos. Inside the egg, the *Oryzias* embryo is covered by a thick chorion (Figure 2). In the laboratory, I often find that *Oryzias* embryos that have escaped harvesting are in a dry part of the aquarium, such as being on an aquarium pump. After being observed using a microscope, the embryo was still alive and growing. From this phenomenon, it is hypothesized that the *Oryzias* embryo can be transported dry for inter-calibration between ecotoxicology laboratories. Therefore, it is necessary to conduct a study to compare the process of hatching eggs between those reared in the embryo rearing medium (ERM) and dry media (without being immersed in water).

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 CaCl 2 H₂O, 1.63 g MgSO₄, 1 ml of NaHCO₃ (0.25 g/20 ml H₂O)) which ordered from Merck, Germany.

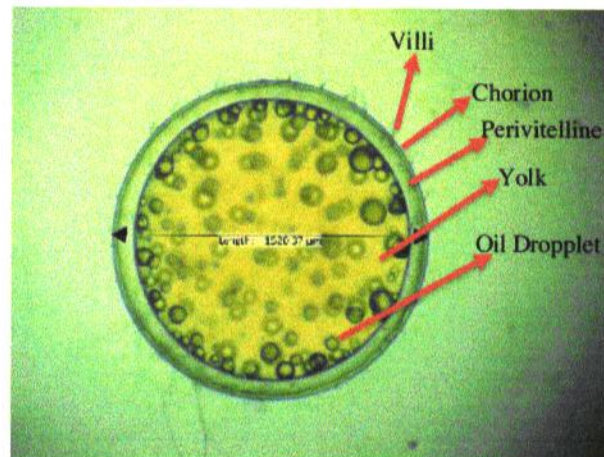
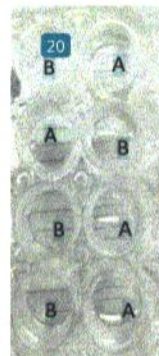
2.2. Eggs production

Although this fish is endemic in Southeast Sulawesi, it is widely sold by ornamental fish traders online. I have purchased the fishes from an online ornamental fish trader. Two pairs of *Oryzias wolasi* were put into the aquarium with five liters of water (Figure 1). The fish are incubated until they produce eggs. During the incubation process, the fish were fed artemia nauplii. This fish can usually spawn a maximum of 10 eggs. However, during rearing in the laboratory, this fish can produce up to 19 eggs.

2.3. The experiment

After three days of feeding artemia nauplii, the broodstocks begin to produce eggs. *Oryzias wolasi* eggs as other *Oryzias* eggs are held together by threads. To remove the threads, the eggs were put into a petri dish containing an embryo rearing media (ERM) solution. The egg clutch was pressed very gently with the finger and rotated until the eggs separate from each other. Afterward, the individual eggs were ready to be used in the experiment.



Figure 1. Broodstock of *Oryzias wolasi*.**Figure 2.** The fertilized *Oryzias wolasi* embryo in stage 1. The egg diameter was 1520.37 μm (magnification 40 x).**Figure 3.** Experimental design.

Eggs that have been separated were put into the microplate with 24 wells individually. The well of this microplate was three ml in volume (Figure 3). Treatment A was treatment with ERM media and B was dry treatment without giving water media. In this experiment, four replications were carried out. The research design can be seen in Figure 3. In this experiment, the eggs in the dry media received water every time an embryo development was observed for about 30 minutes, after which they were in dry condition again. These were more like eggs in nature that experienced metabolic dormancy [15]. The embryo development was observed every 24 hours.

The fish were incubated until the fish hatched. Fish treated with dry media on the seventh day were given ERM with the assumption that on the eighth day the fish will be hatched. Fish of the genus *Oryzias* hatched on days 8-10 [3]. In addition, the dry transport of six days was considered the longest time in transportation. Giving media to fish that were given dry treatment was also intended to prepare embryos for hatching.

2.4. Data analysis

Since data were distributed normally and homogenous, the Student's T-test was used to determine the difference in incubation time, percentage of hatching, and total length of larvae at hatching.

3. Results and Discussion

Oryzias wolasi produces eggs not as many as eggs produced by *O. celebensis*. If *O. celebensis* can produce up to 80 eggs with an average of 25-30 eggs, *O. wolasi* can produce up to 19 eggs with an average of 10 eggs (it was based on my experiment in the laboratory). The eggs that we used during the experiment were produced by one broodstock, totaling nine eggs. A part of the nine eggs, the eight were used in the experiment, so that each treatment contained four eggs. In contrast to *O. celebensis* eggs, which have a clear white color, *O. wolasi* eggs overtime after producing eggs for the first time, the produced eggs become more orange in color (Figure 4, Figure 5).

The broodstock of *O. wolasi*, like the broodstock of the genus *Oryzias*, performs unique external fertilization and lays the fertilized egg under its abdomen (Figure 4). The males of *O. celebensis*, *O. javanicus*, and *O. wolasi* when they were ready to mate (4-5th gonad maturity) were indicated by the brighter the body colors. In addition, the colors of the stripes on the bodies, such as on the caudal fin, are more contrasted so that they appear clearer. The anal fin looks even blacker (Figure 6). *O. wolasi* which ready to mate, the bodies of the male fish become darker (Figure 7).



Figure 4. Broodstock carries eggs under its abdomen.

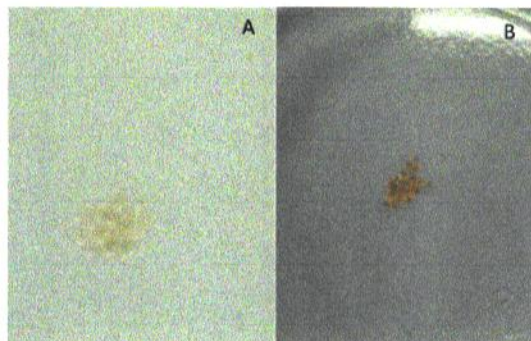


Figure 5. The difference in color of *Oryzias wolasi* eggs when it first produced eggs (A) and after producing eggs five times (B).

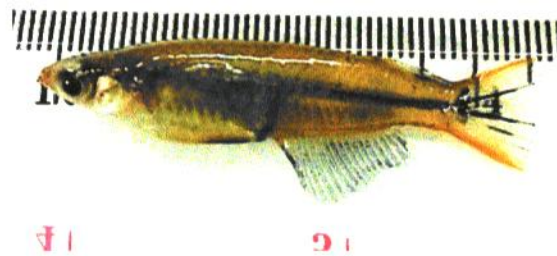


Figure 6. Male *Oryzias celebensis* ready to mate.

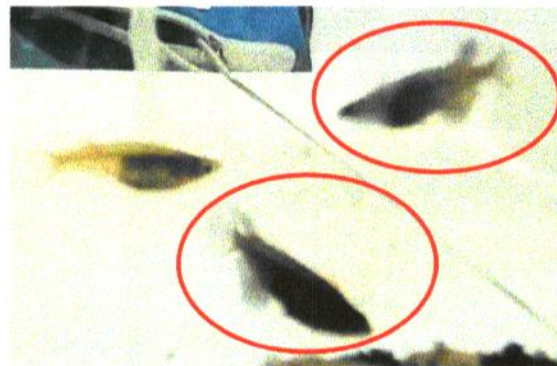


Figure 7. *Oryzias wolasi* ready to mate. Inside the red circle are male fish ready to mate.

Male fish that are ready to mate are aggressive and approach female fish that are ready to perform external fertilization. This approach is part of the introductory process. After a period of introduction to the female, the male fish will continue to approach the female until finally, the male fish hugs the back of the female with his dorsal fin. This process is carried out to make the fertilization process more effective and efficient [16]. The fertilization process is usually performed from morning (8.00) to noon (14.00).

In the fertilization process, there are fertilized (active) eggs and some that are not (inactive). The process of activating *Oryzias* eggs is strongly influenced by pH and the presence of Ca ions in the water [17]. A fertilized egg is characterized by a separation between the chorion and yolk which creates a perivitelline space (Figure 2).

From the experience of cultivating *Oryzias celebensis*, it was found that when fish eggs were not transferred to the egg-rearing media, eggs were stuck in the aquarium pump machine that was not submerged (in dry condition) for days. After the egg was observed under a microscope, it was found that it was still alive. This phenomenon is called metabolic dormancy [15]. From this phenomenon, we tried to investigate whether the egg could be incubated in the dry condition in the laboratory. The

results of this experiment are particularly important in terms of inter-calibration programs for ecotoxicological studies that require a transfer of eggs from one laboratory to another which takes several days.

From the results of the experiment, it was known that there were no significant differences in the incubation time of eggs to hatching between those reared with ERM and dry (mean 12.25 days) (

Figure 8). Also, the two treatments have a 100% hatching percentage. This is probably because *Oryzias* is a fish that uses the K strategy from an ecological perspective. Animals using the K strategy usually have a small number of eggs but those eggs are equipped with the ability to live in better health than those with the R strategy. Therefore, eggs produced by animals with the K strategy must have a high hatching percentage and larval viability. For this purpose, fish from the genus *Oryzias* equip their eggs with thick and hard chorions so that they are resistant to environmental stressors [18,19]. In addition, other functions of chorions such as attracting sperm, activating spermatozoa and as a physical barrier to prevent polyspermy at fertilization [18]. Shortly after fertilization before the chorion thickens, the eggs absorb water causing ultrastructural and cytochemical changes [20]. Chorion hardening is caused by alveolar colloids, Ca^{2+} ions, phospholipids, and enzymes in the chorion layer in glycoproteins or enzymes present in the perivitelline [21,22]. The thick and hard layer of chorion prevents the eggs from breaking easily and is also resistant to high levels of drought [18]. Chorion also serves to block the diffusion of unwanted molecules so that if the diffusion of molecules into the embryo occurs, the diffusion process will be very slow [20]. For that purpose, the chorion is reinforced with an extraembryonic membrane layer under the chorion layer [19]. The hardness of the chorion not only protects against physical disturbances but also protects it from bacterial infection [20,23].

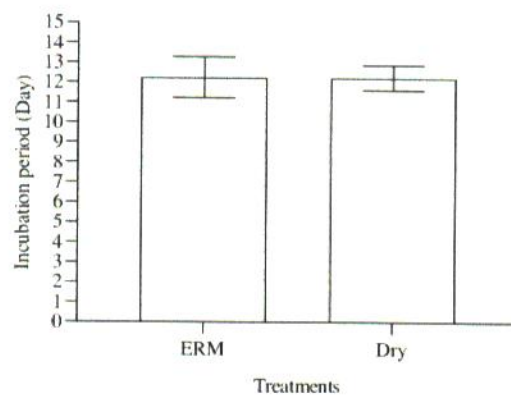


Figure 8. The incubation period between embryos reared in ERM and dry media. There was no significant difference between the two statistically [$p > 0.05$].

Tesoriero suggested that the species *O. latipes* chorion is a product of the oocyte and the mechanism of chorion secretion appears to involve synthesis in the cytoplasm at a concentration in the Golgi apparatus and then small dense vesicles transfer the chorionic precursor material to the developing chorion [24]. However, according to Kinoshita and co-workers, the constituent substances of chorion may come from the liver and oocytes, and chemically, those from oocytes have not been proven [18]. This was confirmed by Murata who performed cloning and cDNA analysis for the choriogenin gene (chorionic precursor) and confirmed that choriogenin gene expression occurred exclusively in the liver and not in the ovaries [25,26]. However, through analysis of cDNAs encoding, it was found that *Oryzias latipes* eggshell glycoproteins originated from oocytes [27]. Choriogenin is

classified into choriogenin [25], choriogenin H [26] and choriogenin I minor [28]. In addition, Hamazaki and co-workers stated that the glycoproteins that make up the inner layer of the chorion come from the liver [29]. Thus, it can be concluded that the chorion of *Oryzias* originates from the liver and oocytes. For biomonitoring, choriogenin has been used as a biomarker against endocrine disruption chemicals [30,31].

The results of the current study revealed that the length at hatching between the embryos reared in ERM media and dry showed significantly different (Figure 9). Embryos from dry media had a mean total length (mean 4.48 mm) longer than those reared in ERM (mean 4.18 mm). Rearing in dry media is imitating the metabolic dormant behavior of *Oryzias* in nature which protects their offspring [15] by laying eggs in terrestrial areas from predators. Besides being able to avoid predators, embryos that are in the terrestrial zone do not need to overcome the diffusion of harmful molecules into the embryo, so the energy to deal with harmful substances is diverted for growth. As we know that naturally, the embryo that is in the water medium experiences the diffusion of small molecules into the embryo which takes place slowly because it is inhibited by the embryonic layer below the chorion [19]. The existence of this inhibitory effort shows that these molecules are not needed by the embryo in the process of growth. In other words, all the substances needed by the embryo are already available in the egg, input from outside the egg will only interfere with the development of the embryo inside the egg. Therefore, when eggs are in a waterless condition, the embryo can use the maximum energy for its growth, so that its growth is faster than those in water media. In addition, the embryo in dry media does not experience fluid stress, so the energy used to overcome the stress is also diverted for growth.

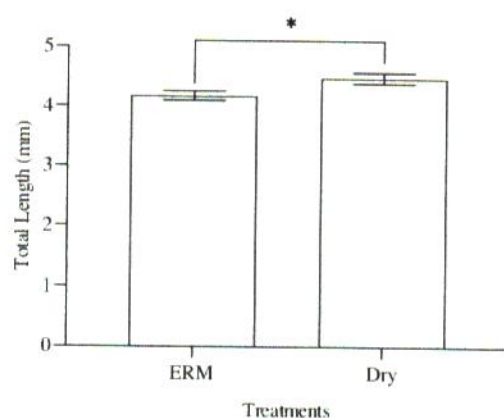


Figure 9. The difference in total length of newly hatched larvae reared in ERM and dry media. Asterisk showed a significant difference between the two treatments [$p < 0.005$].

4. Conclusion

This study showed that the eggs of *Oryzias wolasi* that were kept in dry media had an incubation period that was not significantly different from those reared in ERM. Interestingly, embryos reared in dry media had a longer total length at hatching than those reared in ERM media. Thus, it can be concluded that the embryos of *O. wolasi* which have thick chorion can be transported dry for various studies, especially for ecotoxicological studies. However, more detailed research needs to be done to determine how long can *O. wolasi* embryos live and develop in completely dry media, because, in the

current study, the embryos still received watery conditions when observations of embryos were performed every 24 hours.

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